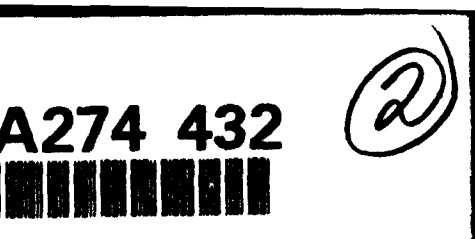

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PRINCIPAL INVESTIGATORS; Edward J. Calabrese, Ph.D.
Paul T. Kostecki, Ph.D.

CONTRACTING ORGANIZATION: University of Massachusetts
Munson Hall
Amherst, MA 01003

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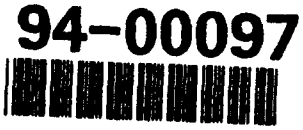

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Paul T. Kostecki, Ph.D.

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Munson Hall
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This study extends the in vivo and in vitro evaluation of peroxisome proliferators in rainbow trout and medaka. In vitro studies with primary cultures of rainbow trout and medaka hepatocytes showed these species to be weak responders to peroxisome proliferators as measured by increased fatty acyl-CoA oxidase activity. Studies conducted in vitro also showed that the peroxisome proliferating agents displayed relatively low capacity to induce a mitogenic response as measured by tritiated thymidine uptake in primary cultures of rainbow trout and medaka hepatocytes. Similar results were shown with rainbow trout in vivo in which no statistically significant increases in liver-to-body weight ratio nor hepatic DNA content were observed following exposure to a known rodent liver mitogen (lead). Hepatic ornithine decarboxylase (ODC) levels were measured in trout and individual medaka and the activity was shown to be 100-200-fold higher in these fish models compared to the rodent. In addition, cell-to-cell communication was characterized by dye-coupling for the first time in vitro with rainbow trout hepatocytes and was shown to be adversely affected by the known tumor promoter TPA. A Project Summary is also included.

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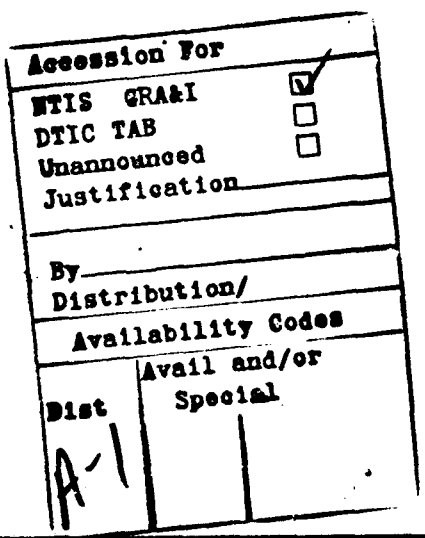

Edward J Calabrese 5/18/93

PI - Signature Date

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PART 1: ORNITHINE DECARBOXYLASE (ODC) ACTIVITY IN THE LIVER OF RAINBOW TROUT (ONCHORYNCHUS MYKISS) AND MEDAKA (ORYZIAS LATIPES)

INTRODUCTION

Polyamines play an important role in the regulation of cellular development and differentiation (Tabor and Tabor, 1984). Ornithine decarboxylase (ODC) catalyzes the rate-limiting step in the polyamine biosynthesis pathway (Russell and Snyder, 1969; Snyder and Russell, 1970; Raina et al., 1976) and shows a rapid and dramatic increase in response to many different growth stimuli (Russell et al., 1976). Increased ODC activity has been observed in regenerating liver (Russell and Snyder, 1969; Hanne and Raina, 1968; Holtta and Janne, 1972; Walker et al., 1978) and has been associated with hepatic tumor promotion in rats (Olson and Russell, 1979). In addition, an increase in hepatic ODC has been observed in rats following single or multiple exposures to the peroxisome proliferators clofibrate (Russell, 1971; Eliassen and Osmudsen, 1984; Fukami et al., 1986) and TCA (Parnell et al., 1988).

To date, ODC activity in fish has been characterized in only two species, the European sea bass (Dicentrarchus labrax L.) (Corti et al., 1987, 1988) and the goldfish (Carassius auratus) (Davalli et al., 1989, 1990). The purpose of this research was to determine hepatic ODC levels in rainbow trout (Onchorynchus mykiss) and medaka (Oryzias latipes) as part of an overall project to assess factors affecting liver carcinogenesis in this model.

Initial methods development was directed towards developing

the spectrophotometric assay for ODC (Ngo et al., 1987) which appeared to be a relatively inexpensive, nonradioactive, yet sensitive measure of ODC activity. However, this proved to be unsuccessful and all subsequent experiments involved $^{14}CO_2$ trapping based on the method of Davis and Paulus (1983).

The initial experiment with $^{14}CO_2$ trapping was conducted on primary cultures of rainbow trout hepatocytes since studies with this *in vitro* system were concurrently being conducted in our laboratory. These encouraging preliminary results prompted further verification of this methodology with the rodent model. After documentation of *in vivo* ODC induction in the rat, an attempt was made to induce ODC *in vivo* in rainbow trout. Based on the lack of response observed in this pilot study, combined with the large interindividual variation previously documented and the possibility of a seasonal effect, it was decided to focus on medaka as the animal model.

Preliminary studies were conducted on pooled medaka livers which were successful in measuring ODC activity. Efforts were then directed towards methods development for determination of ODC activity in individual medaka livers. The results of this investigation were striking in that the ODC activity in individual medaka was determined to be approximately 100-fold greater than seen in rodent liver.

MATERIALS AND METHODS

Spectrophotometric Assay Development

Animals

Male Wistar rats, aged 5 months, were obtained from Charles River Breeding Laboratory, Wilmington, MA. Rats were housed singly at 72 ± 2°F on a 12 hour light/dark cycle and fed Purina Rodent Chow and tap water ad libitum. Male and female rainbow trout (Onchorynchus mykiss) (300 - 350 grams) were obtained from the Massachusetts Division of Fish and Wildlife and maintained in raceways at the Division's Sunderland Fish Hatchery.

Treatment

Rats were injected i.p. with 750 mg/kg chloroform (Fisher Scientific Pittsburgh, PA) to induce ODC activity (Savage et al., 1982) for use as a positive assay control. Animals were sacrificed 18 hours after chloroform administration, their livers were removed, and assayed for putrescine content as described below.

Sample Preparation

Livers were homogenized in a buffer containing 0.1 M NaH_2PO_4, 0.8 mM pyridoxal-5-phosphate, 2.0 mM EDTA, pH 7.5 (Savage et al., 1982). In an attempt to reduce background interference, samples were centrifuged at 30,000 X g for 20 minutes at 4°C. Further efforts of sample purification were made by passing the samples through a series of successively smaller (5, 0.8, 0.45, and 0.22

micron) filters followed by three passages through a Sephadex G25 column.

Ornithine Decarboxylase Assay

Liver supernatants were assayed spectrophotometrically for ODC activity by the method of Ngo et. al. (1983) which measures the amount of putricine generated from ornithine by ODC. 200 ul of substate solution containing (final concentration) 2.5 mM B-mercaptoethanol, 1.5 mM EDTA, 75 nM pyridoxal-5-phosphate and 3 mM l-ornithine HCl in 150 mM potassium phosphate (pH 7.1) was inculated with 100 ul of the liver supernatant for 30 minutes at 37°C. The reaction was stopped by the addition of 200 ul 10% trichloroacetic acid and generated puricine was extracted with 1-pentanol, reacted with 2,4,6-trinitrobenzenesulfonic acid (TNBS) and read spectrophotometrically at 420 nm. Results were calculated by comparison to a standard curve generated with known amounts of putrecine (Table 1).

$^{14}CO_2$ Trapping Method

Animals

Male Fisher 344 rats (180 - 275 grams) were obtained from Charles River Breeding Laboratory, Wilmington, MA. Rats were housed singly at 72 ± 2°F on a 12 hour light/dark cycle and fed Purina Rodent Chow and tap water ad libitum. Male and female rainbow trout (300 - 350 grams) were obtained as described above. Two lots of mixed sexes medaka (Oryzias latipes), 8 months in age, were obtained from Carolina Biological Supply Company, Burlington,

NC. Medaka were maintained at 22°C on a 12 hour light/dark schedule and were fed twice daily alternately with Tetra Fin Flake Food and Tetra Brine Shrimp Treat (Tetra Sales, Inc., Morris Plains, NJ).

Chemicals

DL-a-(difluoromethyl) ornithine hydrochloride monohydrate (DFMO) was a gift of Marion Merrill Dow Research Inst., Cincinnati, OH. L[1-^{14}C] ornithine (50-60 mCi/mol) was purchased from New England Nuclear, Boston, MA. Clofibric acid and diethylnitrosamine (DEN) were obtained from Sigma Chemical Co., St. Louis, MO. All other chemicals were obtained from Sigma Chemical Co., St. Louis, MO unless otherwise stated.

Hepatocyte Isolation and Culture Conditions

Hepatocyte isolation and culture conditions were as previously described (Donohue et al., 1993). Hepatocytes were isolated from sexually mature male rainbow trout (Oncorhyncus mykiss), 300-500g, obtained from the Massachusetts Division of Fisheries and Wildlife fish hatchery in Sunderland, Massachusetts. The hepatocyte isolation was based in the two-step hepatic portal perfusion for fish (Moon et al.,1985; Lipsky et al., 1986) with slight modification. Collagenase concentration was increased form 0.01 to 1.0%. The isolated cells were washed twice by centrifugation at 35g and resuspended in Leibowitz L-15 medium, pH 7.6 supplemented with 10 mM Hepes, 100 U/ml penicillin, 100 ug/ml streptomycin, 2.5

ug/ml amphotericin B, 1 ug/ml insulin (Lipsky et al., 1986; Maitre et al., 1986: Kocal et al., 1988) and 10 uM hydrocortisone Mitchell et al., 1984). Viability, determined by trypan blue exclusion, was 95 ± 3%. Six million cells were plated onto 60 mm culture dishes (Fisher Scientific Pittsburgh, PA) and allowed to attach and spread for 4 hours prior to treatment.

Treatment

In Vitro Study: A stock solution of clofibric acid was prepared in dimethylsulfoxide (DMSO) and added to the medium to give final concentrations of 2.75 mM clofibric acid and 0.4% DMSO. Vehicle control cultures were exposed to medium containing 0.4% DMSO. Insulin was omitted from selected cultures to test for the effect of this hormone on ODC induction.

In Vivo Studies: To induce ODC activity for use as a positive assay control, rats were injected i.p. with 750 mg/kg chloroform (Savage et al., 1982) or 100 mg DEN/kg 1:9 mixture of emulphor (Emulphor EL-620, consisting of 1M castor oil + 30 M ethylene oxide, GAF Corporation, Wayne, NJ) and 0.9% saline (Olson and Russell, 1979). Rats were sacrificed 18 hours after chloroform or DEN administration, their livers were removed and processed as described below. Trout were injected i.p. with 100 or 200 mg DEN/kg in 0.5 ml emulphor/saline mixture (1:9). Fish were sacrificed 24 and 48 hours after DEN administration, their livers were removed and processed as described below.

Sample Preparation

In Vitro Study: Samples were taken at 0, 2, 5, and 24 hours of exposure. Hepatocytes were dislodged from culture dishes with a rubber policeman and transferred to centrifuge tubes. Following centrifugation at 35 X G, cells were resuspended in 235 ul homogenizing buffer (50 uM EDTA, 20 uM pyridoxal-5-phosphate, 2.5 mM dithiothreitol, 25 mM Tris-HCl, pH 7.2) containing 1% Triton X-100, transferred to microcentrifuge tubes and set on ice for 10 minutes. Samples were then centrifuged at 6000 Xg for 10 minutes at 4°C. Assay was run on the supernatants.

In Vivo Studies: Rats were sacrificed by CO_2 gas. Rainbow trout and medaka were sacrificed by exposure to 3-aminobenzoic acid ethyl ester (MS-222) (Sigma Chemical Company, St. Louis, MO). Livers were removed and homogenized in a 0.1 M phosphate buffer pH 7.2 containing 0.1 mM pyridoxal-5-phosphate, 2.0 mM EDTA, 5 mM dithiothreitol (DTT) and 100uM phenylmethylsulfonyl fluoride (PMSF). Homogenates were centrifuged at 15,000 X G for 30 minutes.

Ornithine Decarboxylase Assay

Supernatants were assayed for ODC by the method of Davis and Paulus (1983) which measured the amount of $^{14}CO_2$ liberated from L[1-^{14}C] ornithine. The final concentration of reagents in the enzyme assay mix was 100 mM potassium phosphate buffer pH 7.2, 100 uM PMSF, 5 mM DDT, 1 mM EDTA, 5 mM pyridoxal-5-phosphate, 2 mM L-ornithine and 5 uCi L[1-^{14}C] ornithine (59 mCi/mmol). DL-a-(difluoromethyl) ornithine hydrochloride monohydrate (DFMO) was

added to some samples at a final concentration of 5 mM. $^{14}CO_2$ was collected on 1 X 2 cm pieces of Whatman #1 filter paper spotted with 10 ul of monoethanolamine-methylcellusolve (1:2, v/v). 200 ul of the enzyme assay mixture was added to 100 ul of enzyme and incubated at 37°C for 60 minutes. The reaction was stopped by the addition of 200 ul 10% trichloroacetic acid. Tubes were incubated for additional 90 minutes at 37°C to collect the $^{14}CO_2$. Filter papers were counted in Ecoscint A fluor (National Diagnostics, Manville, NJ) on a 1217 Rackbeta liquid scintillation spectrophotometer (LKB Instruments, Inc., Gaithersburg, MD). Protein content of the samples was determined by the BCA Protein Assay Kit (Pierce Chemical Co., Rockford, IL).

Statistical Analysis

The statistical package STATISTIX (Version 3.5, Analytical Software, St. Paul, MN) was used for data analysis. Data were analyzed by one- and two-way ANOVA to detect treatment and treatment and time effects ($\alpha < 0.05$). In cases where there was only a control and one treatment group, a Student's t-test was used ($\alpha < 0.05$). Multiple regression analysis was performed to detect linear trends for generation of standard curves.

RESULTS

Spectrophotometric Assay Development

A standard curve of putrescine concentrations ranging from 5 uM to 250 uM was created (r^2 = 0.9808, r = 0.9903) (Table 1). We were unable to obtain accurate levels of putrescine in trout or chloroform-treated rat liver due to the high background color of the sample blank (Table 2). Attempts to reduce the background color by filtration followed by passage through a Sephadex G25 column were unsuccessful (Table 3).

$^{14}CO_2$ Trapping Method

In Vitro Study: Preliminary results indicate that we are able to detect ODC in control and clofibric acid-treated cultures of rainbow trout hepatocytes (Table 4). However, we were unable to perform a two-way ANOVA because the pattern of missing values (i.e., inconsistent numbers of data points at various time points and treatments) made it impossible to derive estimates of the missing values so that interpretation of the ANOVA results would be meaningful.

In Vivo Studies: Preliminary results with chloroform-treated rats showed a statistically significant increase in ODC activity in animals exposed to chloroform compared to control animals (Student's t-test results: t = -11.17, p = 0.0067) (Table 5). Although a 10-fold increase in ODC activity over untreated controls was observed in the DEN-treated rats, it was not statistically

significant due to the small number of experimental animals per treatment group (n = 3) (Student's t-test results: t = -2.50, p = 0.0668) (Table 6).

Preliminary results with 4 untreated rainbow trout demonstrated a wide range of mean individual values, ranging from 0.025 to 0.195 nmoles $^{14}CO_2$/hr/mg protein (Table 7). When ODC activity was analyzed by sex in a subsequent experiment, no statistically significant difference was observed (ANOVA results: F = 1.27, p = 0.3022) (Table 8). Hepatic ODC activity in rainbow trout was shown to be inhibited by DFMO (Student's t-test results: t = 2.76, p = 0.0398) (Table 9). There was no statistically significant treatment effect in rainbow trout treated with DEN on ODC activity at 24 or 48 hours when analyzed by treatment and time (ANOVA results: effect of treatment on ODC, F = 0.73, p = 0.5775; effect of time on ODC, F = 2.95, p = 0.3354; effect of treatment and time on ODC, F = 1.03, p = 0.4935) (Table 10). However, it should be noted that a 5-fold increase in ODC activity over untreated controls was observed at 48 hours in the 200 mg DEN/kg group. This was not statistically significant due to the small number of trout per treatment group (n = 2). Preliminary data show a statistically significant difference in hepatic ODC activity in trout by season, with ODC levels observed in October ranging from 10- to 100-fold less than those observed in May and June of the same year (Student's t-test results t = 4.02, p = 0.0008) (Table 11). It is also possible that a day effect is operating since a 10-fold difference in ODC activity was observed on two days in May

(May 21 and 23) and in June (June 11 and 13) (Table 11). ODC activity was linear over time (Figure 1).

Preliminary results of ODC activity in pooled medaka livers are presented in Table 12. The mean ± SE ODC activity was 1.25 ± 0.39 nmoles $^{14}CO_2$/hr/mg protein. This activity was also inhibited by DFMO (Student's t-test results: t = 3.18, p = 0.0130) (Table 12). Subsequent studies done on individual medaka livers are presented in Table 13. The mean ± SE hepatic ODC activity for all the fish was 1.69 ± 0.26 nmoles $^{14}CO_2$/hr/mg protein (Table 13). There was considerable interindividual variation in the fish ranging from a low of 0.06 to a high of 8.43 nmoles $^{14}CO_2$/hr/mg protein (i.e., a range of 140-fold). There was also a statistically significant difference between the two lots of fish (Student's t-test results t = 2.11, p = 0.0418). The mean ODC activity of lot 1 was 2.21 ± 0.42 nmole $^{14}CO_2$/hr/mg protein while the mean ODC activity of lot 2 was 1.17 ± 0.26 $^{14}CO_2$/hr/mg protein. The mean ODC activity of the males was 1.60 ± 0.33 nmoles $^{14}CO_2$/hr/mg protein and the mean ODC activity in the females was 1.84 ± 0.41 nmoles $^{14}CO_2$/hr/mg protein. The difference in hepatic ODC activity between male and female medaka was not statisically significant (Student's t-test results t = 0.45, p = 0.6581). ODC activity was linear over time (Figure 2).

Table 1: Absorbance values at 420 nm of putrescine concentrations generated to create a standard curve with the spectrophotometric assay for ODC (Ngo et al., 1987).

Concentration of Putrescine (umoles)	Absorbance at 420 nm
5	0.023
5	0.015
10	0.029
10	0.075
25	0.094
25	0.108
75	0.301
75	0.315
125	0.517
125	0.451
250	1.007
250	1.203

Table 2: Preliminary results showing hepatic putrescine concentrations in male rainbow trout and male chloroform-treated Wistar rats.

Animal Model	Treatment	Putrescine Concentration (nm/4hr/mg protein) Sample	Sample Blank
Rat	Untreated	3.06	2.94
	Untreated	2.90	
	Chloroform	3.54	3.05
	Chloroform	3.61	
Trout	Untreated	4.54	4.64
	Untreated	4.37	
	Untreated	3.07	5.09
	Untreated	3.56	

Table 3: Results of purification attempt to reduce background interference observed in the spectrophotometric assay based on putrescine concentrations.

	Absorbance at 420 nm		
Purification Step	Sample	Sample Blank	Sample - Sample Blank
Crude homogenate	1.540	1.348	0.192
5 micron filtration	1.419	1.618	-0.199
0.8 micron filtration	1.446	1.361	0.085
0.45 micron filtration	1.531	1.507	0.024
0.22 micron filtration	1.610	1.745	-0.135
First pass Sephadex G25	1.632	1.605	0.027
Second pass Sephadex G25	1.293	1.442	-0.149
Third pass Sephadex G25	1.587	1.644	-0.057

Table 4: ODC activity measured by $^{14}CO_2$ trapping at 0, 2, 5, and 24 hours of exposure to 2.75 mM clofibric acid in primary cultures of female rainbow trout hepatocytes.

Treatment	ODC Activity[1]			
	0 hr	2 hr	5 hr	24 hr
Control	1.1 ± 0.1 (4)	2.4 ± 0.4 (4)	2.4 ± 0.6 (3)	2.0 ± 0.6 (3)
DMSO Control		1.4 ± 0.1 (3)	1.2 (1)	1.1 ± 0.0 (2)
Control - Insulin		1.2 ± 0.1 (4)	2.0 ± 0.2 (2)	2.3 ± 0.5 (3)
Clofibric Acid		1.9 ± 0.5 (4)	2.0 ± 0.1 (3)	2.2 ± 0.4 (4)

[1] Activity is expressed as nmoles $^{14}CO_2$/hr/mg protein; values = mean ± SE; ( ) = number of cultures.

Table 5: Hepatic ODC activity in male Fisher 344 rats 18 hours after i.p. injection of 750 mg chloroform/kg.

Treatment	n	ODC Activity[1] nmoles $^{14}CO_2$/hr/mg protein
Control	3	0.016 ± 0.007
Chloroform	3	0.072 ± 0.005 *

[1] Values = mean ± SE.
* Statistically significant from control as analyzed by Student's t-test, t = -11.17, p = 0.0067.

Table 6: Hepatic ODC activity in male Fisher 344 rats 18 hours after i.p. injection of 200 mg DEN/kg.

Treatment	n	ODC Activity[1] nmoles $^{14}CO_2$/hr/mg protein
Control	3	0.003 ± 0.005
DEN	3	0.033 ± 0.001

[1] Values = mean ± SE.

Table 7: Preliminary hepatic ODC activities in untreated rainbow trout.

Trout #	ODC Activity[1] nmoles $^{14}CO_2$/hr/mg protein	
1	0.035	
	0.024	mean = 0.025
	0.024	SEM = 0.004
	0.017	
2	0.160	
	0.265	mean = 0.195
	0.160	SEM = 0.035
3	0.057	
	0.060	mean = 0.058
	0.056	SEM = 0.001
4	0.028	
	0.023	mean = 0.027
	0.027	SEM = 0.002
	0.032	

[1] Three or four replicate samples were run on each trout.

Table 8: Hepatic ODC activities in male and female rainbow trout.

ODC Activity nmoles $^{14}CO_2$/hr/mg protein	
Males	Females
0.879	0.408
2.500	0.457
2.490	1.325
3.530	3.290

There was no statisically significant difference in ODC activity between male and female trout.
(Student't t-test t = -1.13, p = 0.3022)

Table 9: Hepatic ODC activities in untreated rainbow trout in the presence and absence of the inhibitor DFMO.

ODC Activity[1]	
-DFMO	+DFMO*
1.380	0.028
9.360	0.010
4.010	0.001
4.190	0.002
2.050	0.001
0.534	0.000

[1] Activity is expressed as nmoles $^{14}CO_2$/hr/mg protein.
* Statistically significant from control as analyzed by Student's t-test, t = 2.76, p = 0.0398.

Table 10: Preliminary hepatic ODC activities in untreated and DEN-treated rainbow trout 24 and 48 hours post i.p. injection.

Treatment	ODC Activity[1] nmoles $^{14}CO_2$/hr/mg protein 24 hours	48 hours
Control	2.5 ± 0.4	3.8 ± 0.6
DEN 100 mg/kg	2.7 ± 1.2	1.5 ± 0.2
DEN 200 mg/kg	1.7 ± 0.1	11.4 ± 7.3

[1] Values = mean ± SE; n = 2.

Table 11: Hepatic ODC activities in untreated rainbow trout by date.

Date	n	ODC Activity nmoles $^{14}CO_2$/hr/mg protein
5/21/91	3	0.39 ± 0.13
5/23/91	4	3.51 ± 1.46
6/06/91	4	0.77 ± 0.21
6/11/91	4	0.26 ± 0.12
6/13/91	4	2.95 ± 0.27
10/30/91	6	0.03 ± 0.01*
10/31/91	4	0.03 ± 0.01*

*ODC activity for October are statisically significantly different from ODC activity in May and June. Student's t-test t = 4.02, p = 0.0008.

Table 12: Effect of DFMO on ODC activity (nmoles $^{14}CO_2$/hr/mg protein) in pooled medaka livers.

Sample Number	ODC Activity	
	Without DFMO	With DFMO*
1[a]	1.48	0.06
2[a]	2.32	0.00
3[a]	3.74	0.00
4[b]	0.93	0.00
5[b]	0.07	0.00
6[b]	1.40	0.00
7[b]	0.58	0.00
8[b]	0.65	0.00
9[b]	0.10	0.00

[a] Each sample represents a pool of four medaka livers.
[b] Each sample represents a pool of two medaka livers.
* Statistically significant from control (without DFMO) as analyzed by Student's t-test, $t = 3.18$, $p = 0.0130$.

Table 13: Mean ODC activity (nmoles $^{14}CO_2$/hr/mg protein) in individual medaka.

	Sample Size	Mean	Standard Error of the Mean
ODC in medaka: mixed sexes; lots 1 & 2	48	1.69	0.26
ODC in female medaka: lots 1 & 2	17	1.84[1]	0.41
ODC in male medaka: lots 1 & 2	31	1.60[1]	0.33
ODC in medaka: mixed sexes; lot 1	24	2.21[2]	0.42
ODC in female medaka: lot 1	9	2.36	0.61
ODC in male medaka: lot 1	15	2.11	0.57
ODC in medaka: mixed sexes; lot 2	24	1.17[2]	0.26
ODC in female medaka: lot 2	8	1.26	0.48
ODC in male medaka: lot 2	16	1.12	0.32

[1]ODC activity of male and female medaka was not statisically significantly different as analysed by Student's t-test, $t = 0.45$, $p = 0.6581$.

[2]ODC activity of lots 1 and 2 was statistically significantly different as analysed by Student's t-test, $t = 2.11$, $p = 0.0418$.

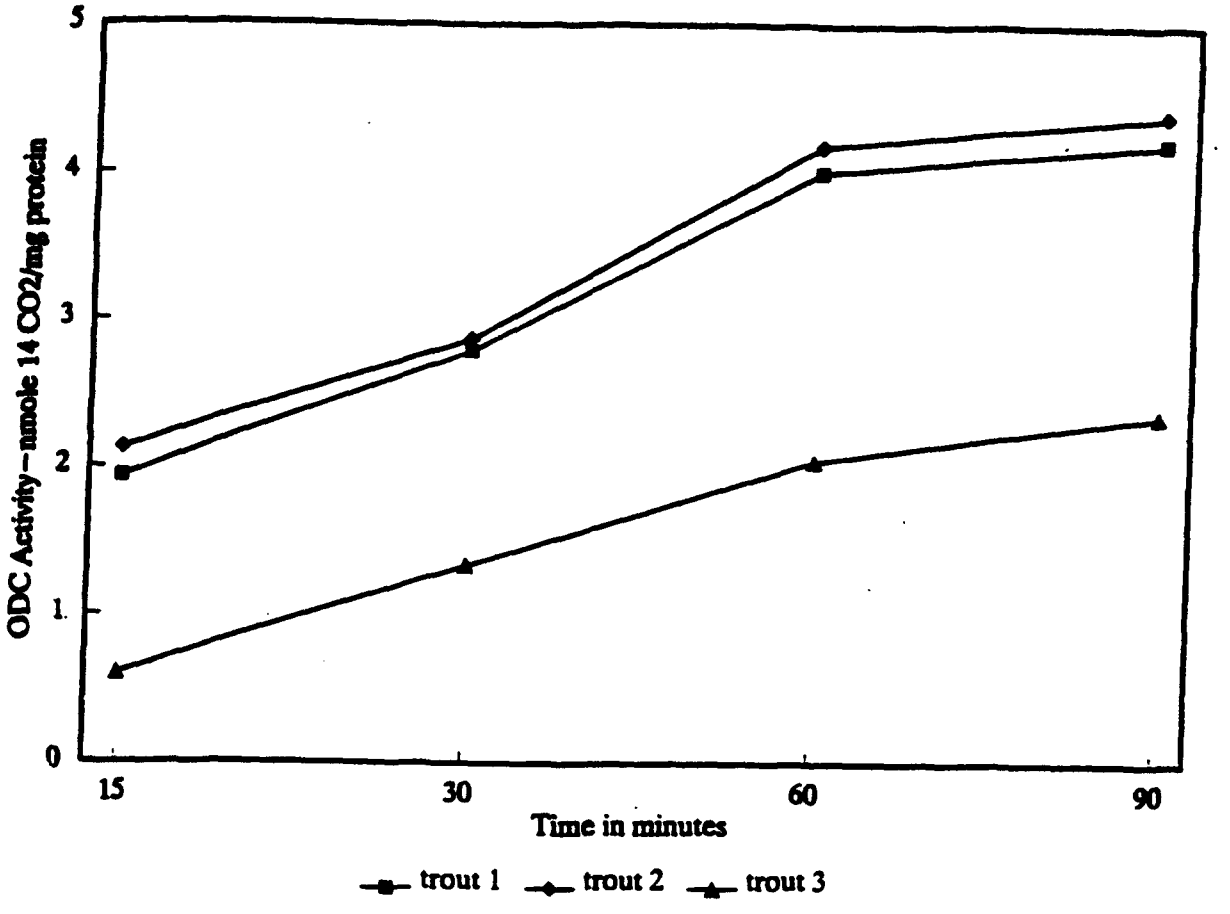

Figure 1: ODC activity (nmoles $^{14}CO_2$/mg protein) in rainbow trout livers over time. Linear regression analysis of data over 15 - 60 minutes yielded: r = 0.9950 for trout #1; r = 0.9980 for trout #2; r = 0.9350 for trout #3.

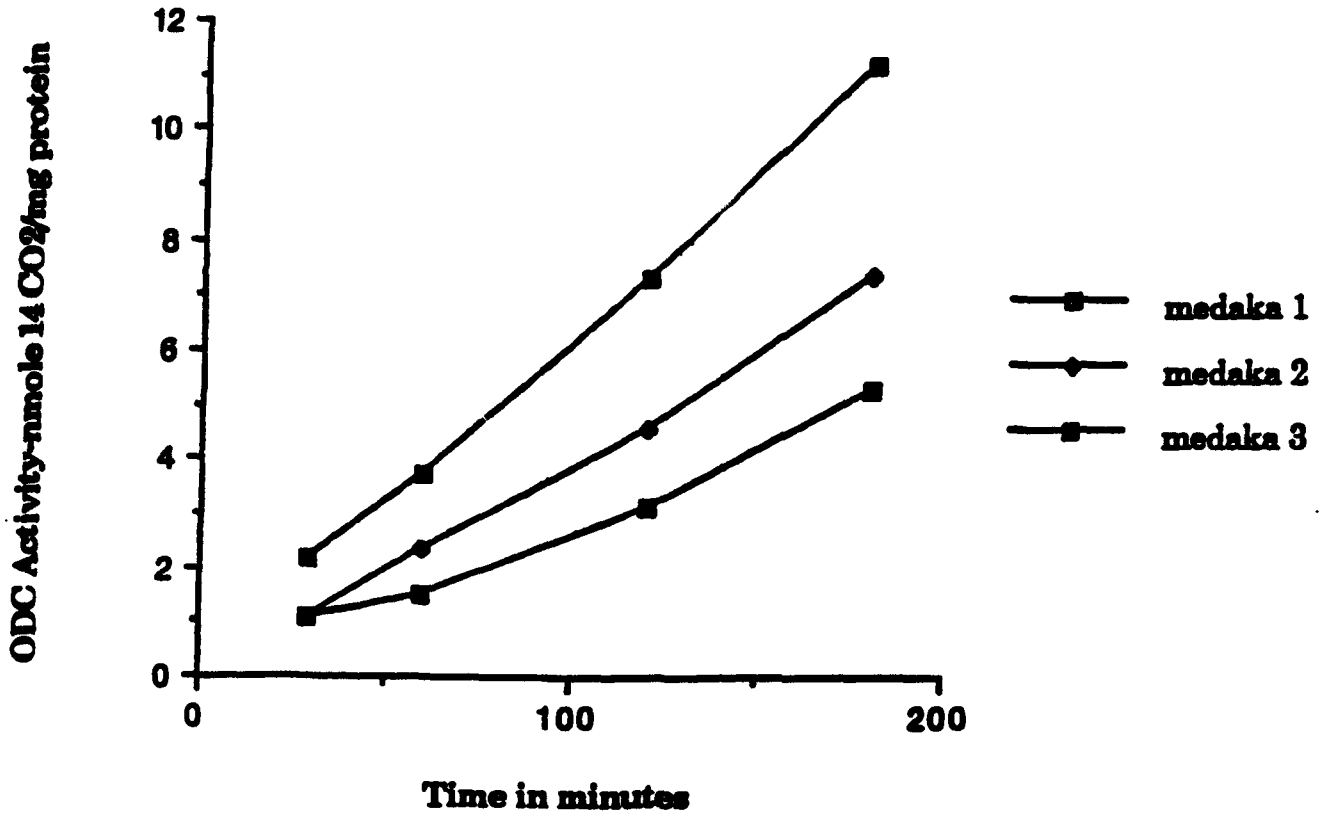

Figure 2: ODC activity (nmoles $^{14}CO_2$/mg protein) in pooled (4 livers/sample) medaka liver samples over time. Linear regression analysis of data over 30 - 180 minutes yielded: r = 0.9856 for medaka #1; r = 0.9958 for medaka #2; r = 0.9968 for medaka #3.

DISCUSSION

Our initial efforts directed towards developing he spectrophotometric assay for ODC (Ngo et al., 1987) were unsuccessful when applied to the analysis of ODC in tissue. The arguments for use of this assay were based on results obtained from purified ODC and commercially available ornithine and putrescine, and did not address the issue of verification or applicability of this method to tissue samples. Due to the nonspecific formation of colored TNBS-adducts in these samples, it is questionable whether further efforts should be directed in this area.

Subsequent efforts based on the $^{14}CO_2$ trapping method (Davis and Paulus, 1983) were successful and represent the first documentation of hepatic ODC activity in rainbow trout and medaka. The data with rainbow trout show a large interindividual variation which does not appear to be due to sex (Student's t-test, $t = -1.13$, $p = 0.3022$) (Tables 7-10). In addition, a seasonal effect may also be an important factor in the activity of rainbow trout hepatic ODC activity (Table 11). A seasonal effect has been shown in goldfish (Devalli et al., 1989, 1990). Additional studies need to be conducted over the course of a year to verify these initial observations.

The results with individual medaka livers also demonstrated a large standard deviation which appears to be due to interindividual variation and not to the sex of the animal (Student's t-test, $t = 0.45$, $p = 0.6581$) (Table 13). It should be emphasized that these findings were reported for individual medaka which represents a

significant improvement over our earlier attempts utilizing pooled samples of up to four fish. The capacity to obtain reliable data on individual fish will provide both significantly improved opportunities to understand chronic disease processes in this model species and assistance in the statistical design and planning of future investigations.

Compared to rats, rainbow trout have higher hepatic ODC activity by factors of 10 to 100, depending on the individual and the season, and medaka have higher activity by a factor of 100 (Fukami et al., 1986; Olson and Russell, 1979; Savage et al., 1982). This elevated level of ODC activity in both species was not a procedural artifact as demonstrated by its linear response over time (Figures 1 and 2) and its inhibition by DFMO (Tables 9 and 12).

Other investigators have demonstrated higher activity of hepatic ODC in fish compared to rats (Corti et al., 1987; Devalli et al., 1989, 1990), although not to the extent observed in medaka. Sea bass, in a comparison study with rats, were shown to have higher levels of hepatic ODC by a factor of approximately 3 (Corti et al., 1987). Goldfish exhibit maximum hepatic ODC activity, subject to seasonal variation, higher than rats by a factor of approximately 10 (Devalli et al., 1989, 1990).

The capacity to inhibit ODC with DFMO will provide an opportunity to assess experimentally the role of ODC in tumor promotion and progression. This has been exploited with great success in both rodent and human carcinogenesis (Danzin et al.,

1979; Sjoerdsma and Schechter, 1984). Similar use in fish could yield significant insights concerning features controlling the process of hepatocarcinogenesis.

The significance of elevated activity of normal hepatic ODC in medaka is at present unknown, although it has been suggested that ODC and polyamines are involved in adaptive metabolic responses to environmental changes or stress stimuli (Devalli et al., 1990). Clarification of the relationship of elevated levels of hepatic ODC activity to both normal metabolic processes and to the susceptibility of medaka to chemically-induced hepatocarcinogenesis is strongly recommended.

CONCLUSIONS

Normal hepatic ODC activity has been determined for the first time in rainbow trout and individual medaka. A large interindividual variation was observed in both species which was not related to sex. Preliminary data suggest that there may be a seasonal effect on hepatic ODC activity in rainbow trout. ODC activity in rainbow trout ranged from approximately 10-fold to 100-fold greater than in normal rat liver. ODC activity observed in individual medaka was approximately 100-fold greater than that observed in normal rat liver.

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PART 2: RAINBOW TROUT IN VIVO MITOGENTICITY STUDY

INTRODUCTION

The role of cell proliferation from a variety of mitogenic stimuli has become a major interest in the field of chemical carcinogenesis. Cell proliferation resulting from direct mitogenic stimulation has been considered to be more closely related to hepatic tumor promotion than peroxisome proliferation in the epigenetic carcinogen hypolipidemic drugs such as clofibrate and ciprofibrate (Bieri et al., 1986).

Lead has been employed as a chemical to produce a potent mitogenic response in the rodent liver in order to assess the role of mitogenicity in chemically induced hepatocarcinogenicity (Columbano et al., 1984; Ledda-Columbano, 1983). The present study was designed to assess if the rainbow trout, a model extensively used in fish carcinogenesis studies (Lee et al., 1968; Sinnhuber et al., 1968, 1974; Hendricks et al., 1980; Shelton et al., 1984; Rawlowski et al., 1985; Bailey et al., 1988), would also be sensitive to lead as a mitogen.

These experiments extend the scope of the pilot study by 1) incorporating higher dosages of lead nitrate (i.e., increasing dosages from 125 to 250 and 375 mg/kg), 2) quantifying the hepatic DNA content, 3) including lead acetate as a control for the salt form of lead, and 4) adding i.v. administration as a second route of exposure.

MATERIALS AND METHODS

Animals

Rainbow trout (Onchorynchus mykiss) were obtained from the Massachusetts Division of Fisheries and Wildlife fish hatchery in Sunderland, MA. The trout were maintained at this facility during experiments in 1,100 liter fiberglass tanks with a fresh water flow-through exchange rate of 110 liters per minute. Trout ranged in age and weight from 18 to 24 months and approximately 80 to 400 grams, respectively. Fish were separated into individual tanks by treatment group and fed a standard diet of trout feed (Zeigler Brothers, Gardners, PA) prior to, but not during, experiments. Water temperatures ranged from 8°C to 11°C during experiments.

Chemicals

Lead nitrate and lead acetate were obtained from Sigma Chemical Company, St. Louis, MO. Lead acetate was also administered to ensure that any response observed was not due to the chemical form of salt used.

Treatment

Both intravenous (i.v.) and intraperitoneal (i.p.) injections of lead nitrate were administered to account for potential variation in results due to route of exposure. Trout were anesthesized in water containing 100 ppm 3-aminobenzoic acid ethyl ester (MS-222) for dosing. Lead nitrate and lead acetate were

dissolved in distilled water and filter sterilized. A final volume of 0.2 ml was injected via a 26 gauge needle into the peritoneal cavity through the center region of the ventral side of the trout. Intravenous injections consisted of 0.1 ml volume of lead nitrate solution administered through a 26 gauge needle into the jugular vein at the base of the first gill arch. Controls received equal volumes of distilled water only. Equal numbers of control fish were sacrificed each time fish from treatment groups were sacrificed.

Liver-to-Body Weight Ratio

Wet body weight was measured at the end of the study period. Livers were removed at termination and weighed. Data were expressed as grams of liver/100 grams body weight.

Hepatic DNA Content

Hepatic DNA content was measured by the diphenylamine method of Schneider (Schneider, 1957). Data were expressed as mg DNA/100 grams body weight. A standard curve was generated ($r^2 = 0.9909$, $r = 0.9954$) (Table 14).

Statistical Analysis

The statistical package STATISTIX (Version 3.5, Analytical Software, St. Paul, MN) was used for data analysis. Data were analyzed by one-way ANOVA to detect treatment effects ($\alpha < 0.05$). For comparison of liver-to-body weight ratio between the control

and treatment group in the 115 hour i.v. lead nitrate study (Table 15) Student's t-test was used ($\alpha < 0.05$). Multiple regression analysis was performed to detect linear trend for generation of standard curve.

RESULTS

Liver-to-Body Weight Ratio

A single intraperitoneal injection of lead nitrate or lead acetate did not result in any statistically significant changes in liver-to-body weight ratio after 64 hours ($F = 0.214$, $p = 0.8850$) (Table 16). The effect of a single intravenous injection of lead nitrate yielded similar results at 72 hours ($F = 1.312$, $p = 0.2997$) and at 115 hours ($t = -1.70$, $p = 0.1198$) (Table 15).

Hepatic DNA Content

No statistically significant change in hepatic DNA content was observed in treatment groups 64 hours following a single i.p. injection of lead nitrate or lead acetate ($F = 0.189$, $p = 0.9019$) (Table 16).

Table 14: Absorbance values at 600 nm of DNA concentrations generated to create a standard curve for determination of hepatic DNA content.

DNA Concentration ug/ml	Absorbance 600 nm
10	0.014
10	0.031
25	0.040
25	0.038
50	0.069
50	0.076
75	0.104
75	0.110
100	0.131
100	0.146
150	0.195
150	0.202

Table 15: Effect of a single intravenous injection of lead nitrate on liver-to-body weight ratio[1] in rainbow trout after 72 and 115 hours.

Treatment	Dose (mg/kg)	Liver-to- Body Weight Ratio 72 hour	115 hour
Control	0	1.20 ± 0.09	1.30 ± 0.07
Lead nitrate	0.5	1.27 ± 0.15	
Lead nitrate	1.0	1.23 ± 0.04	
Lead nitrate	5.0	1.48 ± 0.14	1.35 ± 0.08

[1]Values = mean ± SE; units = grams of liver/100 grams body weight; n = 6.

Table 16: Effect of lead nitrate and lead acetate on liver-to-body weight ratio and DNA content from a single intraperitoneal injection after 64 hours in rainbow trout.

Treatment	Dose mg/kg	Liver-to-Body Weight Ratio[1]	Hepatic DNA Content[2]
Control	0	1.50 ± 0.13	3.32 ± 0.22
Lead nitrate	250	1.39 ± 0.09	3.23 ± 0.18
Lead nitrate	375	1.41 ± 0.23	3.30 ± 0.28
Lead acetate	375	1.40 ± 0.11	3.48 ± 0.24

[1]Values = mean ± SE; units = grams of liver/100 grams body weight; n = 5.
[2]VAlues = mean ± SE; units = mg DNA/100 grams body weight; n = 5.

DISCUSSION

The preliminary data showed no statistically significant increase in liver-to-body weight ratio in rainbow trout administered a single i.p. injection of lead nitrate (25, 50, 75, 100, or 125 mg/kg) at 2, 3, 4, or 5 days following exposure (Bell, et al. 1993). The subsequent studies reported in this document not only confirm the earlier results, but also demonstrate that increased dosages (Table 16) and an alternative route of exposure (i.e., i.v. injection) (Table 15) do not effect the outcome.

The inability of lead to induce cell proliferation or hyperplasia in rainbow trout was in contrast to the Wistar rat model of Columbano (1983, 1985). A single intravenous injection (100 umole/kg body weight) resulted in a substantial increase in the liver-to-body weight ratio (i.e., increased by 70% within 48 - 72 hours). The increased liver-to-body weight ratio is sustained for several days until the process of apoptosis is initiated resulting over the next several days in a dramatic decrease in liver cell number such that by days 7 - 10 the liver-to-body weight ratio returns to normal.

This study provides evidence of significant interspecies variation in the response of rainbow trout and Wistar rats to mitogenic stimuli such as lead. To what extent this differential response is related to susceptibility to chemically induced carcinogenicity remains to be explored.

CONCLUSION

The inability of lead to induce cell proliferation in the rainbow trout is in marked contrast to its effect on the Wistar rat.

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PART 3: THE EFFECT OF PEROXISOME PROLIFERATORS ON S-PHASE SYNTHESIS IN PRIMARY CULTURES OF FISH HEPATOCYTES

INTRODUCTION

Cell proliferation has been implicated as a mechanism important to tumor promotion and can result from direct mitogenic stimulation (Butterworth et al., 1987). A number of agents that have the capacity to cause peroxisome proliferation have also been shown to have mitogenic properties in rat hepatocytes (Bieri et al., 1986, 1987; Marsman et al., 1988). It has been demonstrated that peroxisome proliferation and replicative DNA synthesis can be stimulated independently and that the mitogenic potency of these substances cannot be predicted from their effects on the peroxisomal compartment (Bieri et al., 1987).

The in vivo and in vitro inducibility of peroxisome proliferation in rainbow trout is considerably less pronounced than that observed in rodents (Henderson and Sargent, 1983; Yang, 1990; Yang et al., 1990; Scarano, 1991; Donohue et al., 1992). Since the carcinogenic potency of peroxisome proliferators has been more closely associated with their mitogenic potency than with their capacity for peroxisome proliferation (Bieri et al., 1986, 1987a,b; Marsman et al., 1988), it is important to clarify this response in aquatic species to better understand the carcinogenic process in such organisms. The effect of peroxisome proliferators on DNA synthesis under defined conditions of primary culture is one approach that can be used in the characterization of this type of

nongenotoxic carcinogen.

Kocal et al. (1988a) suggested that trout hepatocytes *in vitro* rapidly catabolize thymidine to a product that is not incorporated into DNA, thereby invalidating the use of ^{3}H-thymidine as a marker of DNA synthesis under certain conditions. Preliminary studies were successfully designed to overcome this limitation by conducting 2 hour-pulse labelling during periods of expected DNA synthesis to maintain a concentration of ^{3}H-thymidine sufficient to mark cells in S-phase over time. Data from these experiments demonstrated that a wave of replicative DNA synthesis occurs between 18 and 30 hours post plating in both rainbow trout and medaka heaptocyte cultures. In addition, treatment related effects in S-phase synthesis were able to be detected.

In the present study, seven structurally diverse peroxisome proliferators were tested for their mitogenic potency in primary hepatocyte cultures of rainbow trout or medaka. Lead nitrate, a known liver mitogen in rats (Columbano et al., 1983, 1985; Ledda-Columbano, 1983), was selected as a positive control since the *in vivo* verification of positive results *in vitro* was considered possible (Part 2 of this report).

MATERIALS AND METHODS

Animals

Rainbow trout (*Onchorynchus mykiss*) weighing 300-350 grams were obtained from the Massachusetts Division of Fisheries and Wildlife fish hatchery in Sunderland, MA. Medaka (*Oryzias*

latipes), 8 months in age, were obtained from Caroline Biological Supply Company, Burlington, NC.

Chemicals

Ciprofibrate was obtained from Sterling Research, Rennselaer, NY. Lead nitrate, lead acetate, gemfibrozil, clofibric acid and trichloroacetic acid (TCA) were purchased from Sigma Chemical Company, St. Louis, MO. Nafenopin was obtained from CIBA-GEIGY Corp., Summit, NJ. Mono-2-ethylhexyl phthalate acid ester (MEHP) was obtained from American Tokyo Kasei, Inc., Portland, OR. 2,4-dichlorophenoxyacetic acid dimethyl amine (2,4-D) was purchased from Dow Chemical Co., Midland, MI.

Hepatocyte Isolation

Rainbow trout: Hepatocytes were isolated by collagenase perfusion based on the procedure for rodents (Seglen, 1973) and modified for fish (Moon et al., 1985; Lipsky et al., 1986; Maitre et al., 1986; Kocal et al., 1988b). In order to facilitate the perfusion technique, the perfusion was not done in situ. Trout were anesthesized with MS-222 (3-aminobenzoic acid ethyl ester, Sigma Chemical Co.), placed on a surgical board and incised from the urogenital pore to the gills. The bile was removed from the gall bladder with a 1 cc tuberculin syringe. The liver was then separated from the gall bladder and transferred to a sterile petri dish. The sinus venosus was cannulated using a blunt 18 gauge needle and ligated. Initially the liver was perfused with 10 mM

HEPES buffer, pH 7.4, containing 136.9 mM NaCl, 3.4 mM KCl, 0.4 mM KH_2PO_4, 4.1 mM $NaHCO_3$, 5.5 mM glucose, 0.1 mM EGTA, followed by perfusion with the same buffer withoug EGTA, but containing 1 mg/ml collagenase (Type II, Worthington Biochemical Corp., Freehold, NJ) and 5.1 mM $CaCl_2$. During perfusion with the dissociating solutions the liver was gently massaged to increase both blood clearance and hepatocyte yield (Moon et al., 1985). At the end of the perfusion, the liver was washed with HEPES buffer and the hepatocyes dissociated using a sterile pipet and forceps. The hepatocytes were passed sequentially through sterile 500, 250, 150, and 75 gauge Nitex screening (Tetko, Inc., Elmsford, NY). The cell suspension was then centrifuged for two minutes at 35 X g. The pellet was resuspended in culture medium and centrifuged for two minutes at 35 X g. The final pellet was resuspended in culture medium. Viability was determined by trypan blue exclusion test and only prepartions with 95% or more viability were utilized.

Medaka: Medaka were anesthesized with MS-222, transferred to a disecting tray and incised from the urogenital pore to the gills. The liver was removed, separated from the gall bladder and transferred to a sterile beaker containing 2 ml of 0.5% trypsin, 5.3 mM EDTA (Gibco Laboratories, Grand Island, NY), reconstituted in HEPES buffer, pH 7.4, containing 136.9 mM NaCl, 3.4 mM KCl, 0.4 mM KH_2PO_4, 4.1 mM $NaHPO_3$, and 5.5 mM glucose, and stirred for 10 minutes at room temperature. The cell suspension was transferred to microcentrifuge tubes and centrifuged for 2 minutes at 35 X g. The pellets were resuspended in culture medium and centrifuged for

2 minutes at 35 X g. The final pellet was resuspended in culture medium. Viability was determined by the trypan blue exclusion test and only preparations with 95% or more viability were utilized.

Culture Conditions

Chemicals were obtained from Gibco Laboratories, Grand Island, NY, unless otherwise stated. The culture medium, based on previous trout studies (Lipsky et al., 1986; Maitre et al., 1986; Kocal et al., 1988b) consisted of Leibowitz L-15, 10 mM HEPES, 100 U/ml penicillin, 100 ug/ml streptomycin, 2.5 ug/ml amphotericin B (Sigma), and 1 ug/ml insulin, pH 7.6. Trout hepatocytes, plated in 0.2 ml medium at a density of 7 X 10^4 cells/chamber in an 8-chamber Permanoz slide (Baxter Scientific Products, McGraw Park, IL), were incubated at 15°C in 100% air. Medaka hepatocytes, plated in 0.2 ml medium at densities dependent on liver size of 1.6 to 18.0 X 10^4 cells/chamber, were incubated at 22°C in 100% air. Plating density was less than confluent to reduce cell-to-cell contact and optimize S-phase response (Nakamura and Ichihara, 1985). Stock solutions of agents were prepared in dimethy sulfoxide (DMSO) and added to the medium to give a final DMSO concentration of 0.4%. The concentrations of ciprofibrate, clofibric acid and gemfibrozil used with rainbow trout hepatocytes were the concentrations shown to induce the highest acyl-CoA oxidase activities <u>in vitro</u> as previously described (i.e., 1.00 mM, 2.75 mM, and 1.25 mM, respectively) (Donohue et al., 1993). All other agent concentrations for rainbow trout and medaka were determined in

preliminary dose-range finding experiments (Table 17). The highest concentrations that did not show toxicity by trypan blue exclusion following 48 hours exposure were chosen. Control cultures were exposed to medium containing 0.4% DMSO without the agent. Treatment was initiated 4 hours after plating.

Replicative DNA Synthesis

Replicative DNA synthesis was assessed based on the procedures of Klaunig (1984) and Kelly and Maddock (1985). Pulse labelling was conducted 18-30 hours following exposure to test chemicals during which time medium containing ^{3}H-thymidine (10 uCi/ml; 50 Ci/mmol; New England Nuclear, Boston, MA) was changed every two hours. Preliminary studies with lead nitrate demonstrated a 1.9-fold increase in cumulative percentage of cells in S-phase over control in the 18-30 hour time period. Less dramatic treatment related increases were observed in the 8-18 and 34-44 hour time periods that gradually decreased over time to 68 hours. 10 mM hydroxyurea, a specific inhibitor of replicative DNA synthesis (Lammers and Follman, 1983) was added to the medium of selected cultures two hours prior to treatment with ^{3}H-thymidine (Bieri et al., 1988). At the end of this time, hepatocytes were washed in Hanks Balanced Salt Solution (HBSS) containing 1 mM thymidine, followed by two washes with HBSS. Cells were fixed with methanol at -20°C for 10 minutes (Baserga, 1989). Slides were air dried, coated with NTB-3 emulsion (Eastman Kodak Co., Rochester, NY) diluted 1:1 with distilled water, and kept in a light-tight box at

-20°C for 12 days. S-phase synthesis was determined by dense nuclear labelling and the cumulative percentage of cells in S-phase was quantified by counting 1000 consecutive morphologically unaltered hepatocytes in each of three replicative chambers for each treatment. To verify that the DNA synthesis was replicative and not repair, these results were compared to the number of cells in S-phase synthesis observed in cultures pretreated with hydroxyurea.

Statistical Analysis

The statistical package STATISTIX (Version 3.5, Analytical Software, St. Paul, MN) was used for data analysis. Data were analyzed by one-way ANOVA ($\alpha < 0.05$) for trout and by a paired t-test ($\alpha < 0.05$) for medaka due to the difference in their respective study designs.

Initially, a pilot study was conducted with medaka hepatocytes to investigate the possibility of using a balanced incomplete block study design (personal communication Dr. Edward Stanek, Professor of Biostatistics, University of Massachusetts, Amherst; Cochran and Cox, 1957) (Figure 3). This study design was considered since it would reduce the overall number of fish required. However, due to the interindividual variability in the S-phase synthesis response, as demonstrated by a value of 0.13% in the DMSO control culture (Fish #1) compared to 0.07% in the lead nitrate (positive control) culture (Fish #3) (Table 18), it was decided that the study design be changed so that each fish could be used as its own control and

the treatment effect analyzed by paired t-test analysis.

RESULTS

Replicative DNA Synthesis

Rainbow trout: No statistically significant increase in cumulative percentage of cells in S-phase over control was observed in any treatment group (ANOVA results: $F = 1.08$, $p = 0.3944$) (Table 19). The lead nitrate treatment which was to serve as a positive control displayed a response 1.7-fold greater than the control. Treatment with clofibric acid elicited a 1.4-fold increase over control.

Medaka: Statistically signficant increases over control in cumulative percentage of cells in S-phase were observed only in the lead acetate- ($t = -8.00$, $p = 0.0153$) and lead nitrate- ($F = -5.00$, $p = 0.0377$) treated groups (Table 20). Treatment with ciprofibrate approached statistical significance ($t = -3.25$, $p = 0.0831$) with a 1.5-fold increase over control.

Table 17: Preliminary dose-response results for *in vitro* S-phase synthesis study with peroxisome proliferators and rainbow trout or medaka hepatocytes.

Animal Model	Peroxisome Proliferator	Concentration	48 hr % Viability[1]
Rainbow Trout	Control	0 mM	90
	DMSO Control	0 mM	91
	Nafenopin	0.064 mM	89
		0.096 mM	85
		0.160 mM	90
	TCA	1.0 mM	93
		2.5 mM	89
		5.0 mM	90
	2,4-D	1.0 mM	80
		2.5 mM	0
		5.0 mM	0
	MEHP	0.10 mM	93
		0.25 mM	90
		0.50 mM	88
Medaka	Control	0 mM	92
	DMSO Control	0 mM	90
	Clofibric Acid	2.0 mM	87
		3.0 mM	84
		4.0 mM	76
	Ciprofibrate	0.5 mM	87
		1.0 mM	90
		1.5 mM	83
	Nafenopin	0.064 mM	93
		0.096 mM	87
		0.160 mM	90
	Gemfibrozil	0.50 mM	91
		1.00 mM	89
		1.25 mM	87
	TCA	1.0 mM	90
		2.5 mM	92
		5.0 mM	88
	2,4-D	1.0 mM	80
		2.5 mM	0
		5.0 mM	0
	MEHP	0.10 mM	93
		0.25 mM	93
		0.50 mM	90

[1] Viability measured by trypan blue exclusion test.

	Treatment		
Fish #1	1	2	3
Fish #2	4	5	6
Fish #3	7	8	9

Treatments

1 = DMSO Control
2 = 2.750 mM Clofibric Acid
3 = 1.000 mM Ciprofibrate
4 = 1.250 mM Gemfibrozil
5 = 0.160 mM Nafenopin
6 = 5.000 mM TCA
7 = 0.500 mM 2,4-D
8 = 0.500 mM MEHP
9 = 0.001 mM Pb Nitrate

Figure 3: Balanced incomplete block study design for S-phase synthesis pilot study with medaka heptocytes (Cochran and Cox, 1957, p. 376).

Table 18: Results of preliminary incomplete block study design for analysis of S-phase synthesis in primary cultures of medaka hepatocytes.

Fish #	Treatment	Cumulative % Cells in S-phase[1]	X-fold Control
1	DMSO Control	0.13 ± 0.09	
	2.750 mM Clofibric Acid	0.17 ± 0.03	1.3
	1.000 mM Ciprofibrate	0.10 ± 0.00	0.8
2	1.250 mM Gemfibrozil	0.20 ± 0.06	1.5
	0.160 mM Nafenopin	0.37 ± 0.12	2.9
	5.000 mM TCA	0.17 ± 0.03	1.3
3	0.500 mM 2,4-D	0.00 ± 0.00	-
	0.500 mM MEHP	0.07 ± 0.03	0.5
	0.001 mM PbNitrate	0.07 ± 0.03	0.5

[1]Each value represents the mean ± SE cumulative percentage of cells in S-phase of 3 replicate cultures; 1000 hepatocytes were counted in each culture; three treatments were conducted on the hepatocytes from each fish.
Note: Although a one-way ANOVA was conducted on this data ($F = 3.31$, $p = 0.0165$), due to the interindividual variation in response it was decided not to adopt this study design.

Table 19: Cumulative percentage of rainbow trout hepatocytes in S-phase synthesis during the 18-30 hour time period following exposure to test chemical.

Test Chemical	Concentration	Cumulative % Cells in S-Phase[1]	X-Fold Control
Control		0.07 ± 0.02	
Lead Acetate	0.001 mM	0.12 ± 0.03	1.7
Lead Nitrate	0.001 mM	0.12 ± 0.03	1.7
Clofibric Acid	2.750 mM	0.10 ± 0.04	1.4
Ciprofibrate	1.000 mM	0.08 ± 0.03	1.1
Nafenopin	0.160 mM	0.08 ± 0.03	1.1
MEHP	0.500 mM	0.07 ± 0.03	1.0
Gemfibrozil	1.250 mM	0.05 ± 0.03	0.7
TCA	5.000 mM	0.03 ± 0.02	0.4
2,4-D	0.500 mM	0.03 ± 0.02	0.4

[1]The value represents the mean ± SE of six cultures; three cultures from each of two fish on two different days. 1000 hepatocytes were counted in each culture.

Table 20: Cumulative percentage of medaka hepatocytes in S-phase synthesis during the 18-30 hour time period following exposure to test chemicals.

Test Chemical	Conc. mM	Cumulative % Cells in S-Phase[1] Control	Treated	t Statistic	p	X-Fold Control
		0.07	0.13			
Lead Acetate	0.001	0.03	0.07	-8.00	0.0153*	1.9
		0.07	0.13			
		0.07	0.13			
Lead Nitrate	0.001	0.07	0.13	-5.00	0.0377*	1.7
		0.07	0.10			
		0.07	0.10			
Ciprofibrate	1.000	0.10	0.13	-3.25	0.0831	1.5
		0.10	0.17			
		0.07	0.07			
Nafenopin	0.160	0.10	0.17	-1.64	0.2419	1.4
		0.07	0.10			
		0.17	0.27			
Clofibric Acid	2.750	0.13	0.10	-0.98	0.4320	1.3
		0.13	0.17			
		0.07	0.10			
TCA	5.000	0.07	0.07	-1.00	0.4226	1.1
		0.07	0.07			
		0.07	0.03			
MEHP	0.500	0.07	0.10	-0.40	0.7295	1.2
		0.03	0.07			
		0.03	0.03			
2,4-D	0.500	0.03	0.07	0.00	1.0000	1.0
		0.07	0.03			
		0.13	0.07			
Gemfibrozil	1.250	0.13	0.13	1.00	0.4226	0.8
		0.10	0.10			

[1]Each value represents the average cumulative percentage of cells in S-phase of 3 replicate cultures; 1000 hepatocytes were counted in each culture.
*Statistically significant from control ($p < 0.05$) as determined by paired t-test.

DISCUSSION

The present study demonstrates that S-phase synthesis induced by peroxisome proliferators in primary cultures of rainbow trout and medaka hepatocytes does not result in any statistically significant increase over control cultures. However, it is possible that clofibric acid in the trout and ciprofibrate in the medake may stimulate S-phase synthesis, but the sample size precluded a more definitive appraisal. The standard serum-free culture conditions used in this study were designed to promote attachment, viability and function. Hepatic function has been demonstrated by the ability of these cells to metabolize acetaminophen (via cytochrome P-450) and to respond to peroxisome proliferators by induction of acyl-CoA oxidase and the peroxisomal bifunctional enzyme (Donohue et al., 1992). Evidence that the ^{3}H-thymidine incorporation observed was mainly replicative as demonstrated by the 80-90% inhibition of incorportation by 10 uM hydroxyurea.

These in vitro results are supported by in vivo data on liver-to-body weight ratios in rainbow trout exposed to peroxisome. No statistically significant increases in liver-to-body weight ratios were observed in rainbow trout treated via intraperitoneal injection for 2 to 4 weeks with clofibrate, lactofen, DEHP, 2,4-D or TCE (Yang, 1990; Scarano, 1991). In the same studies, statistically significant increases were observed only in the highest dose gemifibrozil group (Scarano, 1991) and only marginally significant increases were observed in the highest dose

ciprofibrate group (Yang, 1990). A seven-week feeding study with trout and high fat diet plus DEHP also revealed no statistically significant increase in liver-to-body weight ratio (Henderson and Sargent, 1983).

Further evidence that rainbow trout do not resond to mitogen stimulation in a manner similar to rodents was shown in the *in vitro* and *in vivo* experiments with lead compounds. Columbano et al. (1983, 1985) demonstrated a significant increase in liver-to-body weight ratios and hepatic DNA content within 48 hours in rats administered a single injection of lead nitrate compared to control rats. No lead-induced increases in liver-to-body weight ratio or hepatic DNA content were observed in trout following a single injection of lead nitrate or lead acetate as previously presented in this report. Despite the fact that lead nitrate and lead acetate *in vitro* showed the highest S-phase response over control compared to the seven peroxisome proliferators, the overall cumulative percentage of cells in S-phase were low.

The present study was designed to address two principal questions: whether trout hepatocytes respond to rodent mitogenic stimuli *in vitro*, and whether selected rodent peroxisome proliferators induce S-phase synthesis in primary cultures of trout and medaka hepatocytes. The data display possible weak responses *in vitro* in both trout and medaka. While either weak or lack of response in the *in vitro* system of the trout and medaka suggests that further efforts should be directed toward optimizing the conditions of the culture system to facilitate the S-phase

response, the lack of an apparant mitogenic response *in vivo* in trout to lead compounds suggests that the trout and medaka may not be sensitive to these mitogenic stimuli.

CONCLUSION

Peroxisome proliferators do not significantly induce S-phase synthesis in primary cultures of rainbow trout or medaka hepatocytes under the conditions described.

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PART 4: EFFECT OF PEROXISOME PROLIFERATORS ON PEROXISOMAL B-OXIDATION IN PRIMARY CULTURES OF RAINBOW TROUT HEPATOCYTES

INTRODUCTION

This study is a continuation of the previously reported *in vitro* data which documented the effect of clofibric acid, ciprofibrate, and gemfibrozil on peroxisomal beta-oxidation in primary cultures of rainbow trout hepatocytes (Donohue et al., 1993). The present work extends the scope of the previous report by evaluating the effect of the peroxisome proliferators nafenopin, 2,4-D, MEHP and TCA on the same system.

MATERIALS AND METHODS

Animals

Sexually mature male and female rainbow trout (*Onchorynchus mykiss*), weighing 300-500 grams, were obtained from the Massachusetts Division of Fisheries and Wildlife fish hatchery in Sunderland, MA.

Chemicals

Nafenopin was obtained from CIBA-GEIGY Corp., Summit, NJ. 2,4-Dichlorophenoxyacetic acid dimethyl amine (2,4-D) was purchased from Dow Chemical Co., Midland, MI. Mono-2-ethylhexyl phthalate acid ester (MEHP) was obtained from American Tokyo Kasei, Inc., Portland, OR. Trichloroacetic acid (TCA) was purchased from Sigma

Chemical Co., St. Louis, MO.

Hepatocyte Isolation

Hepatocytes were isolated as previously described in Part 3 of this report.

Selection of Doses

Effect of vehicle control (0.4% DMSO) and peroxisome proliferators on cell viability were determined from a preliminary screening assay (Table 17, Part 3). The highest concentrations that did not cause severe cytotoxicity at 48 hours in culture (< 85% viability, as judged by trypan blue exclusion) were selected as the maximum experimental concentrations and used to extrapolate downward to five or six lower concentrations.

Culture Conditions

Culture conditions were as previously described in this report. Hepatocytes were seeded at 1 X 10^6 viable cells per 5 ml culture medium in 60-mm petri dishes (Beckton-Dickinson Co., Oxnard, CA) and incubated at 15°C in 100% air. After 5-6 hours, treatment was commenced by removing the culture medium and replacing with medium containing test compound dissolved in dimethylsulfoxide (DMSO, final concentration 0.4% v/v). Every 24 hours thereafter, the medium was removed and the cultures redosed. Three to four culture dishes per treatment level were used. Vehicle control cultures were exposed to 0.4% DMSO (v/v).

Fatty Acyl-CoA Oxidase (FACO) Activity

At the end of 48 hours, cell monolayers were detached from culture dishes using a rubber policeman and centrifuged 4 minutes at 35 X g. Supernatant was discarded and pellet resuspended in 10% sucrose, 3 mM imidazole (SI) buffer. In order to solubilize the cells, Triton X-100 was added at a final concentration of 1%. Suspensions sat on ice for 10 minutes followed by centrifugation for 10 minutes at 6000 X g. Supernatant was saved for analysis. FACO activity was measured spectrophotometrically (Small et al., 1985). Activity was quantified as nmoles DCF oxidized/min/mg protein at 30°C. Protein was determined by the BCA Protein Assay (Pierce Chemical Co., Rockford, IL).

Statistical Analysis

The statistical package STATISTIX (Version 3.5, Analytical Software, St. Paul, MN) was used for data analysis. Data were analyzed by one-way ANOVA and Dunnett's t-test for pairwise comparison of means between treated groups and controls. Multiple regression analysis was performed to detect any linear trends.

RESULTS

Fatty Acyl-CoA Oxidase Activity

Data are presented in Table 21. No statistically significant change in fatty acyl-CoA oxidase activity was observed in cultures exposed to nafenopin (ANOVA results: $F = 1.81$, $p = 0.1853$), MEHP

(ANOVA results: $F = 1.31$, $p = 0.3238$), or TCA (ANOVA results: $F = 0.81$, $p = 0.3008$). A statistically significant treatment effect was observed with 2,4-D (ANOVA results: $F = 21.26$, $p = 0.0000$). Cultures exposed to 1 mM, 3 mM or 5 mM 2,4-D demonstrated a statistically significant decrease in fatty acyl-CoA oxidase activity compared to the vehicle control (Dunnett's t-test results: $t = 3.45$, $p < 0.05$). Multiple regression analysis showed a nearly statistically significant linear trend in the cultures exposed to nafenopin ($F = 4.37$, $p = 0.0528$, $r^2 = 0.1656$, $r = 0.4069$), but no statistically significant linear trends in cultures exposed to MEHP ($F = 0.46$, $p = 0.5053$, $r^2 = -0.0325$, $r = 0.1803$), or TCA ($F = 2.97$, $p = 0.1018$, $r^2 = 0.0941$, $r = 0.3067$). A treatment related linear trend was observed in the cultures exposed to 2,4-D ($F = 71.71$, $p = 0.0000$, $r^2 = 0.7795$, $r = 0.8829$).

Table 21: Effect of nafenopin, 2,4-D, MEHP, and TCA on 48-hour peroxisomal acyl-CoA oxidase activity (FACO)[1] in primary cultures of rainbow trout hepatocytes.

Treatment	FACO	% Vehicle Control
Vehicle Control	3.81 ± 0.40	100
0.016 mM nafenopin	3.70 ± 0.54	97
0.032 mM nafenopin	3.69 ± 0.27	97
0.064 mM nafenopin	4.07 ± 0.75	107
0.096 mM nafenopin	3.69 ± 0.29	97
0.160 mM nafenopin	3.00 ± 0.27	79
Vehicle Control	2.75 ± 0.02	100
0.05 mM 2,4-D	2.10 ± 0.47	76
0.10 mM 2,4-D	2.25 ± 0.45	82
0.50 mM 2,4-D	2.02 ± 0.21	73
1.00 mM 2,4-D	1.85 ± 0.36*	67
3.00 mM 2,4-D	0.75 ± 0.15*	27
5.00 mM 2,4-D	0.62 ± 0.10*	23
Vehicle Control	2.11 ± 0.29	100
0.025 mM MEHP	1.79 ± 0.22	85
0.050 mM MEHP	2.14 ± 0.07	101
0.100 mM MEHP	2.10 ± 0.31	100
0.250 mM MEHP	2.19 ± 0.16	104
0.500 mM MEHP	2.09 ± 0.15	99
Vehicle Control	3.65 ± 1.09	100
0.10 mM TCA	3.56 ± 0.65	98
0.50 mM TCA	3.55 ± 0.75	97
1.00 mM TCA	3.04 ± 0.30	83
3.00 mM TCA	2.95 ± 0.27	81
5.00 mM TCA	3.08 ± 0.26	84

[1]Values = mean + SE nmoles DCF oxidized/min/mg protein, n = 3-4 cultures.
*Statistically significant from respective control as analyzed by Dunnett's t-test comparison of means, $t = 3.45$, $p < 0.05$.

DISCUSSION

Treatment of rainbow trout hepatocytes with nafenopin, 2,4-D, MEHP, or TCA did not induce peroxisomal B-oxidation as measured by increased acyl-CoA oxidase activity at 48 hours (Table 21). Cytotoxicity related to treatment was observed in cultures exposed to 2,4-D which influenced the statistical analysis of this agent. When compared to the vehicle control a statistically significant treatment effect was demonstrated in the cultures exposed to 3 mM and 5 mM 2,4-D (cell viability as measured by trypan blue exclusion = 40% and 25%, respectively). This treatment related cytotoxicity also caused the observed linear dose-response trend in fatty acyl-CoA oxidase activity.

The results presented here are consistent with data obtained from the previous in vitro study (Donohue et al., 1993) and from previous in vivo work (Yang, 1989; Yang et al., 1990) that document the rainbow trout as a weak responder to peroxisome proliferating stimuli. The limited response observed in rainbow trout is similar to that seen in mammals such as marmosets (Holloway et al., 1982), monkeys, dogs (Foxworthy et al., 1990), and humans (Stott, 1988) which are all weak responders when compared to the rodent (Reddy et al., 1973; Foxworthy and Eacho, 1986; Elcombe and Mitchell, 1986; Eacho et al., 1989; Foxworthy et al., 1990; Mitchell et al., 1984; Gray et al., 1983).

CONCLUSION

Treatment of primary cultures of rainbow trout hepatocytes with the peroxisome proliferators nafenopin, 2,4-D, MEHP, and TCA did not induce peroxisomal beta-oxidation as measured by increased acyl-CoA oxidase activity at 48 hours.

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PART 5: PEROXISOME PROLIFERATION POTENTIAL OF HYPOLIPIDEMIC DRUGS IN PRIMARY CULTURES OF MEDAKA HEPATOCYTES

INTRODUCTION

It has been demonstrated that a variety of fish species have hepatic peroxisomes that carry on the beta-oxidation of fatty acids (Kramer et al., 1974; Henderson et al., 1982; Henderson and Sargent, 1984, 1985). Furthermore, the peroxisomal bifunctional enzyme which works as the catalyst for the second and third reactions of the beta-oxidation pathway (Lazarow and deDuve, 1976) has been isolated in rainbow trout (Onchorynchus mykiss) (Baldwin et al., 1990;).

The rainbow trout has been studied for its response to hypolipidemic agents both in vivo (Yang, 1989; Scarano, 1991) and in vitro (Donohue et al., 1993; Final Report). These studies found the rainbow trout to be a poor responder to hypolipidemic drugs.

Data from an in vivo study with medaka (Oryzias latipes) and gemfibrozil (Scarano, 1991) suggests that this species also is a poor responder. However, because of difficulties associated with in vivo work (i.e., such as infection, contamination, and unknown delivered dose) and the success of the previously reported in vitro study with hypolipidemic drugs and rainbow trout hepatocytes (Donohue et al., 1993), primary cultures of medaka hepatocytes were utilized to test the effect of hypolidemic drugs in this species.

In addition, the assay for fatty acyl-CoA oxidase (Small et

al., 1985) was adapted for use with the microplate reader to enable enzyme analysis on individual medaka livers. This eliminated the problems associated with pooling livers and the subsequent statistical analyses.

The hypolipidemic drugs ciprofibrate, clofibric acid, gemfibrozil and nafenopin were used to validate the system as well as to characterize individual medaka's response to peroxisome proliferators *in vitro*.

MATERIALS AND METHODS

Animals

Sexually mature (8 months in age) male and female medaka (*Oryzias latipes*) were obtained from Carolina Biological Corp., Burlington, NC.

Chemicals

Ciprofibrate, clofibric acid, gemfibrozil and nafenopin were obtained as previously described in this report.

Hepatocyte Isolation

Hepatocytes were isolated as previously described in Part 3 of this report.

Culture Conditions

Culture conditions were previously described in this report. Hepatocytes were plated in 0.2 ml medium at densities dependent on the liver size of 1.6 to 84.0 X 10^4 cells/chamber in an eight-chamber permanox slide (Baxter Scientific Products, McGraw Park, IL) and incubated at 22°C in 100% air. Viability was determined by trypan blue exclusion and only preparations with 95% viability or greater were utilized in this study. Stock solutions of agents were prepared in dimethyl sulfoxide (DMSO) and added to the medium to give a final DMSO concentration of 0.4%. Concentrations were determined by preliminary dose-range finding experiments (Table 17, Part 3 of this report). The highest dose that did not show toxicity by trypan blue exclusion following 48 hours of exposure were chosen as the maximum experimental concentrations and used to extrapolate downward to two lower concentrations. Control cultures were exposed to medium containing 0.4% DMSO without the agent. Treatment was initiated 5 hours after plating. Cell viability was determined at the end of 48 hours by trypan blue exclusion (Table 26).

Fatty Acyl-CoA Oxidase (FACO) Activity

The optimum wavelength for determination acyl-CoA oxidase activity is 502 (Small et al., 1985). The microplate reader available for use was equiped with filters of wavelengths 490 and 515. Prior to investing in a filter of wavelenght 500-502 nm a pilot study was conducted using an acyl-CoA oxidase standard (Sigma

Chemical Co., St. Louis, MO) with the spectrophotometer to optimize assay conditions and verify the need for this purchase. The results of this experiment (Table 22) indicate the importance of the 500-502 nm wavelength and assay temperature of 30°C.

Conversion from the spectrophotometer to the microplate reader also necessitated the generation of a standard curve for determination of acyl-CoA oxidase activity (r^2 = 0.9968, r = 0.9984) (Table 23). This defined the limits of detection in this system necessary to ensure the feasibility of measuring the activity in a single medaka liver. In contrast to the set length of the light path (horizontal beam of light; length = width of cuvette) in the spectrophotometer, the length of the light path in the microplate reader can vary slightly with each sample (verical beam of light; length = volume of sample in well). Whereas calculations for the spectrophotometric analysis of acyl-CoA oxidase employ an extinction coefficient dependent upon a fixed light path (i.e., 1 cm), calculations for the microplate reader were extrapolated directly from the standard curve. As a result, since the standard used was defined in terms of units (1 unit of activity forms 1 micromole of H_2O_2/min at 30°C in a peroxidase coupled system; Sigma Chemical Co., St. Louis, MO), the activity of the samples were defined in micromoles (or nanomoles) of H_2O_2/min/mg protein.

Use of the microplate reader enabled a 10-fold reduction in volume of assay solutions and sample. A pilot study in which substrate volumes were adjusted was conducted on untreated male

Wistar rat liver homogenates and isolated medaka hepatocytes to further optimize conditions. The male Wistar rat was selected because it was readily available from a concurrently running study and has known levels of acyl-CoA oxidase activities. These data show that by increasing the substrate (palmitoyl CoA) from 2 ul to 5 ul, detection of acyl-CoA oxidase activity is statistically significantly improved in both the rat (paired t-test results: $t = 3.14$, $p = 0.0165$) and medaka (paired t-test results: $t = 3.95$, $p = 0.0290$) (Table 24).

Preliminary experiments were also conducted to test the retention of acyl-CoA oxidase activity in primary cultures of medaka hepatocytes. Comparison of enzyme activities in isolated hepatocytes (mean $\pm$ SE = 23.2 $\pm$ 7.0 nmoles H_2O_2/min/mg protein) with 48 hour cultures (mean $\pm$ SE = 22.1 $\pm$ 4.2 nmoles H_2O_2/min/mg protein) showed no statistically significant difference (Student's t-test results: $t = 0.12$, $p = 0.9062$) (Table 25).

Therefore, at the end of 48 hours, cell monolayers were detached from culture chambers and processed as previously described for rainbow trout hepatocytes (Part 4, this report). Duplicate samples were analyzed for fatty acyl-CoA oxidase activity at 500 nm with a microplate reader. Assay conditions employed 5 ul of substrate at 30°C. Activity was quantified as nmoles H_2O_2/min/mg protein. Protein was determined by the BCA Protein Assay (Pierce Chemical Co., Rockford, IL) with absorbance read at 595 nm with the microplate reader.

Statistical Analysis

The statistical package STATISTIX (Version 3.5, Analytical Software, St. Paul, MN) was used for data analysis. Data were analyzed by one-way ANOVA to detect the effects of treatment and sex on response ($\alpha < 0.05$). A paired t-test was used to analyze difference in response to the two different assay conditions described (i.e., 2 ul substrate vs. 5 ul substrate) ($\alpha < 0.05$). Student's t-test was employed to detect a difference between 2 groups (i.e., isolated hepatocytes and 48 hour cultured hepatocytes) ($\alpha < 0.05$). Data were also subjected to a multiple regression analysis to determine linear trends.

RESULTS

Data are presented in Table 26 and 26A. No statistically significant change in fatty acyl-CoA oxidase activity was observed in cultures exposed to ciprofibrate (ANOVA results: $F = 0.30$, $p = 0.8241$), clofibric acid (ANOVA results: $F = 0.89$, $p = 0.4668$), gemfibrozil (ANOVA results: $F = 0.62$, $p = 0.6135$), or nafenopin (ANOVA results: $F = 1.08$, $p = 0.3895$) (Table 26). When analyzed by sex (Table 26A), no statistically significant change in fatty acyl-CoA oxidase activity was shown in cultures exposed to ciprofibrate (ANOVA results: $F = 0.95$, $p = 0.3450$), clofibric acid (ANOVA results: $F = 1.19$, $p = 0.2905$), gemfibrozil (ANOVA results: $F = 0.01$, $p = 0.9382$), or nafenopin (ANOVA results: $F = 1.91$, $p = 0.1859$). No treatment related linear trends were observed in cultures exposed to ciprofibrate ($F = 0.07$, $p = 0.7938$, $r^2 =$ -

0.0617, r = 0.2484), clofibric acid (F = 2.02, p = 1.7360, r^2 = 0.0535, r = 0.2313), gemfibrozil (F = 0.22, p = 0.6483, r^2 = -0.0551, r = 0.2347), or nafenopin (F = 3.59, p = 0.0762, r^2 = 0.1324, r = 0.3639).

Table 22: Results of pilot study to verify wavelength and temperature conditions necessary for optimization of microplate reader analysis.

Wavelength nm	Temp. °C	Acyl-CoA Oxidase Standard	
		Change in OD/min	nmoles DCF oxidized/ min/mg protein
490	25	0.0421	2.97
490	30	0.0512	4.74
502	25	0.0696	5.76
502	30	0.0825	7.64
510	25	0.0488	3.87
510	30	0.0749	6.77

Table 23: Change in absorbance over time at 500 nm and 30°C of acyl-CoA oxidase activities generated to create a standard curve for microplate analysis of peroxisomal beta-oxidation in primary cultures of medaka hepatocytes.

Acyl-CoA Oxidase Activity (Units)	Change in Absorbance/min
0.0125	0.0030
0.0125	0.0032
0.0250	0.0062
0.0250	0.0070
0.0500	0.0108
0.1250	0.0310
0.2500	0.0580
0.2500	0.0554

Table 24: Comparison of change in absorbance over time[1] in male Wistar rat liver homogenates and isolated medaka hepatocytes under assay conditions employing 2ul or 5ul substrate (palmitoyl CoA).

Sample	Change in Absorbance/Min at 500 nm	
	2 ul Substrate	5 ul Substrate[2]
Rat	0.0112	0.0152
	0.0126	0.0130
	0.0182	0.0294
	0.0182	0.0336
	0.0040	0.0062
	0.0050	0.0048
	0.0062	0.0184
	0.0074	0.0142
Medaka	0.0100	0.0118
	0.0078	0.0101
	0.0090	0.0144
	0.0052	0.0102

[1] Samples were read at 500 nm and 30°C with the microplate reader.
[2] Statistically significant from 2 ul substrate values when analyzed by paired t-test (rat samples, $t = 3.14$, $p = 0.0165$; medaka samples, $t = 3.95$, $p = 0.0290$).

Table 25: Peroxisomal fatty acyl-CoA oxidase activity in isolated hepatocytes and in 48 hour primary hepatocyte cultures from medaka.

	Activity[1] Units/ml	Protein mg/ml	nmoles H_2O_2 per min/mg protein[2,3]
Isolated Hepatocytes	0.096	2.22	43.2
	0.052	1.67	31.1
	0.005*	1.16	4.3
	0.017	1.56	10.7
	0.045	1.68	27.1
mean ± SE	0.043 ± 0.016	1.66 ± 0.17	23.2 ± 7.0
48 hr Cultures	0.015	0.49	30.2
	0.013	0.83	16.1
	0.024*	1.21	19.9
mean ± SE	0.017 ± 0.003	0.84 ± 0.49	22.1 ± 4.2

[1] Samples were done in duplicate unless indicated with *.
[2] One unit of activity forms 1 micromole of H_2O_2/min at 30°C in a peroxidase coupled system.
[3] There was no statistically significant difference between isolated hepatotocytes and 48 hour cultures (Student's t-test results, t = 0.12, p = 0.9062).

Table 26: Effect of ciprofibrate (Cipro), clofibric acid (Clof), gemfibrozil (Gem), and nafenopin (Naf) on peroxisomal fatty acyl-CoA oxidase activity (FACO) in primary cultures of medaka hepatocytes.

Treatment	Sex[1] M	Sex[1] F	FACO[2,3]	% Vehicle Control	48 hr % Viability[4]
Vehicle Control	3	0	4.13 ± 2.15	100	95
0.33 mM Cipro	3	1	2.65 ± 1.52	64	95
0.67 mM Cipro	3	2	2.75 ± 0.46	67	95
1.00 mM Cipro	4	1	4.40 ± 1.02	107	95
Vehicle Control	2	3	2.69 ± 1.13	100	95
0.50 mM Clof	2	3	4.58 ± 1.38	171	98
1.00 mM Clof	3	1	6.71 ± 2.47	250	98
2.00 mM Clof	5	0	6.30 ± 2.54	235	98
Vehicle Control	2	1	4.26 ± 2.68	100	95
0.33 mM Gem	5	0	2.25 ± 1.57	53	95
0.67 mM Gem	4	0	4.31 ± 1.31	101	95
1.00 mM Gem	3	1	4.19 ± 1.58	98	95
Vehicle Control	3	0	12.10 ± 7.56	100	95
0.033 mM Naf	4	1	9.43 ± 3.65	78	85
0.067 mM Naf	4	1	5.10 ± 2.91	42	85
0.100 mM Naf	4	1	3.12 ± 2.12	26	85

[1] FACO activity by sex is presented in Table 26A.
[2] Values = mean + SE nmoles H_2O_2/min/mg protein, n = 3-5 cultures.
[3] No statistically significant change in FACO activity was observed in cultures exposed to ciprofibrate (ANOVA results: $F = 0.30$, $p = 0.8241$), clofibric acid (ANOVA results: $F = 0.89$, $p = 0.4668$), gemfibrozil (ANOVA results: $F = 0.62$, $p = 0.6135$) or nafenopin (ANOVA results: $F = 1.08$, $p = 0.3895$). No linear trends were observed in cultures exposed to ciprofibrate ($F = 0.07$, $p = 0.7938$, $r^2 = -0.0617$, $r = 0.2484$), clofibric acid ($F = 2.02$, $p = 1.7360$, $r^2 = 0.0535$, $r = 0.2313$), gemfibrozil ($F = 0.22$, $p = 0.6483$, $r^2 = -0.0551$, $r = 0.2347$) or nafenopin ($F = 3.59$, $p = 0.0763$, $r^2 = 0.1324$, $r = 0.3639$).
[4] Viability was determined by trypan blue exclusion.

Table 26A: Effect of ciprofibrate (Cipro), clofibric acid (Clof), gemfibrozil (Gem), and nafenopin (Naf) on peroxisomal fatty acyl-CoA oxidase activity (FACO) in primary cultures of medaka hepatocytes analyzed by gender.

Treatment	FACO[1,2] Males	Females	Both Sexes
Vehicle Control	4.13 ± 2.15 (3)	No Data (0)	4.13 ± 2.15 (3)
0.33 mM Cipro	3.15 ± 2.10 (3)	1.15 (1)	2.65 ± 1.52 (4)
0.67 mM Cipro	3.17 ± 2.33 (3)	2.13 ± 0.54 (2)	2.75 ± 0.46 (5)
1.00 mM Cipro	4.78 ± 1.97 (4)	2.89 (1)	4.40 ± 1.02 (5)
Vehicle Control	3.06 ± 1.98 (2)	2.44 ± 1.70 (3)	2.69 ± 1.13 (5)
0.50 mM Clof	6.56 ± 2.07 (2)	3.27 ± 1.67 (3)	4.58 ± 1.38 (5)
1.00 mM Clof	7.72 ± 3.18 (3)	8.22 (1)	6.71 ± 2.47 (4)
2.00 mM Clof	6.30 ± 2.54 (5)	No Data (0)	6.30 ± 2.54 (5)
Vehicle Control	5.91 ± 2.38 (2)	0.97 (1)	4.26 ± 2.68 (3)
0.33 mM Gem	2.25 ± 1.57 (5)	No Data (0)	2.25 ± 1.57 (5)
0.67 mM Gem	4.31 ± 1.31 (4)	No Data (0)	4.31 ± 1.31 (4)
1.00 mM Gem	3.58 ± 1.17 (3)	6.00 (1)	4.19 ± 1.58 (4)
Vehicle Control	12.10 ± 7.56 (3)	No Data (0)	12.10 ± 7.56 (3)
0.033 mM Naf	11.05 ± 4.23 (4)	2.96 (1)	9.43 ± 3.65 (5)
0.067 mM Naf	6.17 ± 3.48 (4)	0.80 (1)	5.10 ± 2.91 (5)
0.100 mM Naf	3.87 ± 2.57 (4)	0.13 (1)	3.12 ± 2.12 (5)

[1] Values = mean + SE nmoles H_2O_2/min/mg protein, ( ) = n.
[2] When analyzed by sex there was no statistically significant change in FACO activity in cultures exposed to ciprofibrate (ANOVA results: F = 0.95, p = 0.3450), clofibric acid (ANOVA results: F = 1.19, p = 0.2905), gemfibrozil (ANOVA results: F = 0.01, p = 0.9382) or nafenopin (ANOVA results: F = 1.91, p =0.1869).

Table 27: *In vivo* data with pooled medaka livers (Scarano, 1991) converted to nmoles H_2O_2/min/mg protein for comparison with our *in vitro* data (Table 26).

In Vivo Treatment[1]	n	nmoles H_2O_2/min/mg protein[2]
Control (DEHP study)	14	8.0 ± 1.2
Control (TCE study)	10	3.4 ± 1.2
Control (Gem study)	12	9.6 ± 2.6
1.25 ppm Gem	10	10.5 ± 1.8
2.50 ppm Gem	7	8.6 ± 1.5
5.00 ppm Gem	8	10.3 ± 1.9

[1] DEHP = di-(2-ethylhexyl) phthalate; TCE = trichloroethylene; gem = gemfibrozil.
[2] Values represent mean ± SE.

DISCUSSION

Treatment of medaka hepatocytes with ciprofibrate, clofibric acid, gemfibrozil, or nafenopin did not induce peroxisomal beta-oxidation as measured by increased fatty acyl-CoA oxidase activity at 48 hours (Table 26). Cytotoxicity was not a factor as indicated in the 48 hour viability values (Table 26).

The levels of activity measured in isolated hepatocytes and primary cultures of medaka are within the range of detection defined by the standard curve (Table 23). Furthermore, it was shown that this activity was retained in 48 hour cultures (Table 25).

The inability to detect statistically significant changes in acyl-CoA oxidase activity may be due to the large interindividual variation observed within and between experiments (Table 26). Mean control levels between experiments ranged from 2.69 nmoles H_2O_2/min/mg protein in the clofibric acid study to 12.10 nmoles H_2O_2/min/mg protein in the nafenopin study. A similar response was also demonstrated in our in vivo studies (Scarano, 1991) (Table 27) where the mean control levels ranged from 3.4 nmoles H_2O_2/min/mg protein in the TCE study to 9.6 nmoles H_2O_2/min/mg protein in the gemfibrozil study. For comparative purposes, the results of the gemfibrozil study are presented (Table 27) since it is the only hypolipidemic drug evaluated in vivo (Scarano, 1991). No statistically significant gemfibrozil-induced change in acyl-CoA oxidase activity was observed in vivo when analyzed by dose or by sex (Scarano, 1991) which supports our in vitro data analysis.

The results presented here are consistent with previously reported data that document medaka as poor responders to peroxisome proliferating stimuli compared to rodents.

CONCLUSION

Treatment of primary cultures of medaka hepatocytes with the peroxisome proliferators ciprofibrate, clofibric acid, gemfibrozil, or nafenopin did not induce peroxisomal beta-oxidation as measured by increased acyl-CoA oxidase activity at 48 hours.

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PART 6: IN VITRO CELL-TO-CELL COMMUNICATION SYSTEM IN PRIMARY CULTURES OF RAINBOW TROUT HEPATOCYTES

INTRODUCTION

Intercellular communication plays an important role in the regulation of cell growth and differentiation (Trosko et al., 1985). This communication can be mediated by the transfer of ions or small molecules through gap junctions (Loewenstein, 1981). A common property of nongenotoxic carcinogens is the ability to stimulate cell proliferation. Since many nongenotoxic carcinogens also inhibit intercellular communication through gap junctions, it has been hypothesized that their stimulation of cell replication is through the disruption of intercellular communication (Trosko and Chang, 1984).

Much information exists on the effect of tumor promoters on rodent hepatocyte intercellular communication in vivo and in vitro (Ruch and Klaunig, 1986, 1988; Klaunig and Ruch, 1987a, 1987b, 1990). Intercellular communication has been documented by metabolic cooperation in rainbow trout gonadal tissue and fathead minnow epithelial cells (Slater et al., 1983), but no data exist on intercellular communication and tumor promoters in fish hepatocytes.

Gap junction intercellular communication can be monitored by visualizing fluorescent dye transfer between cells (Klaunig and Ruch, 1990). One method (Method 1) involves the exposure of donor

cells to non-polar fluorescein esters in the medium which readily enter the cells where they are hydrolyzed to membrane-impermeable free fluorescein (Sellin et al., 1971, 1974; Rotman and Papermaster, 1966). Diffusion of these dyes through gap junctions into unlabelled recipient cells, subsequently added to the culture, can be monitored by fluorescent microscopy. Method 1 was initially chosen due to the limited instrumentation requirements. However, preliminary work to develop a system based on Method 1 (Sellin et al., 1971; 1974) in primary cultures of rainbow trout hepatocytes was unsuccessful for reasons discussed below.

Another method (Method 2) involves the microinjection of fluorescent dye into individual donor cells and visualization of dye spread (dye-coupling) to adjacent hepatocytes. Method 2 requires the use of specialized instrumentation. Dr. Patricia Wadsworth (Department of Biology, University of Massachusetts, Amherst) donated the use of her laboratory and equipment for the dye-coupling experiments. Instruction in the microinjection technique necessary for preliminary data collection was kindly provided by Dr. Patiricia Wadsworth and Ms Rama Iyengar. This enabled us to characterize, under the defined conditions of primary culture, the onset and duration of gap junction-mediated intercellular communication in rainbow trout hepatocytes. Preliminary experiments with TPA were conducted to investigate the potential use of this model in vitro system for the study of hepatic tumor promotion.

MATERIALS AND METHODS

Animals

Female yearling rainbow trout (Onchorynchus mykiss) 250-300 g were obtained from the Massachusetts Division of Fisheries and Wildlife fish hatchery, Sunderland, MA.

Hepatocyte Isolation

Trout hepatocytes were isolated as previously described in this report.

Method 1: Labelling of donor cells via expsoure in medium.

Chemicals

Flourescein diacetate, was obtained from Sigma Chemical Co., St. Louis, MO. Naphthofluorescein diacetate was purchased from Molecular Probes, Inc., Eugene OR. Fluorescent polystyrene microbeads (0.5 micron diameter) with rhodamine label were obtained from Polysciences, Inc., Warrington, PA. All other tissue culture supplies were obtained from sources previously indicated in this report.

Culture Conditions

Culture conditions were as previously described in this report. Initially donor hepatocytes were seeded at a density of 3 X 10^4 cells/chamber in a two-chamber Permanox slide (Baxter Scientific Products, McGraw Park, IL). Recipient hepatocytes were

seeded at a density of 27 X 10^4 cells/chamber in a different two-chamber Permanox slide (ratio of donor to recipient cells = 1:9). Preliminary experiments attempted to utilize hepatocytes from one perfusion as both donor and recipient populations. However, it was found that once cells were plated they could not be detached and re-plated, which was necessary under this protocol for the recipient population. As a result, donor hepatocytes were isolated from one perfusion and plated. Recipient hepatocytes were isolated from another perfusion performed 24 hours later and were plated directly with the previously attached donor cells. This procedure is based on the methodology used by Klaunig et al. (1989).

Autofluorescence

Autofluorescence of hepatocytes was observed at the fluorescein wavelenght which confounded the results of fluorescein dye transfer. The following experiments were conducted in an attempt to reduce or eliminate this autofluorescence.

Experiment 1: The concentration of fluorscein diacetate in the medium was increased from 5 ug/ml to 25 ug/ml to increase the uptake and resultant signal in the hepatocytes. The fluorescent signal was slightly increased with no increase in cytotoxicity, but not enough to clearly differentiate between dye-transfer and autofluorescence.

Experiment 2: This study was conducted to eliminate possible media-derived fluorescence. Chamber slides with cells were washed five times with phosphate buffered saline (PBS). 0.1% formalin in

PBS was added to the cells and allowed to stand for 10 minutes. Cells were washed twice with PBS, covered with a coverslip, and examined under the microscope. Autofluorescence was not reduced.

Experiment 3: The purpose of this study was to label donor cells with a dye that fluoresces at a wavelength that the cells do not autofluoresce. Naphtholfluorescein diacetate was added to the medium of donor cells at a concentrations of 5, 10 or 50 ug/ml. Cells were checked at 0.25, 1, 4, and 24 hours of exposure to medium containing naphtholfluorescein diacetate. The cells did not take up the label perhaps because this dye is too bulky a molecule for the rainbow trout hepatocyte system (personal communication Molecular Probes, Inc.).

Experiment 4: This study attempted to label donor cells by endocytosis of polystyrene beads labeled with rhodamine, which also fluoresces at a wavelength that the cells do not autofluoresce (personal communication, Dr. James Klaunig, Indiana University School of Medicince, Indianapolis, IN). Cells were exposed to microbeads in medium at a concentration of 100X the concentration of cells (i.e., 3 X 10^6 microbeads to 3 X 10^4 cells/chamber; personal communication Polysciences, Inc.). Additional cultures were set up with microbeads at a concentration of 10X the concentration of cells (i.e., 3 X 10^5 microbeads to 3 X 10^4 cells/chamber) to see if the lower concentration would label adequately. Cells were checked at 1, 3, 6 and 24 hours of exposure to medium containing the beads. The cells did not endocytose the beads at any time point at either concentration.

Therefore, efforts were directed toward microinjection which produces a stronger flurorescent signal this is not confounded by the weaker autofluorescence of the cells.

Method 2: Microinjection of fluorescent dye into donor cells.

Chemicals

Lucifer Yellow CH dye and TPA (12-O-tetradeconoyl phorbol-13-acetate) were purchased from Sigma Chemical Co. (St. Louis, MO). All other tissue culture supplies were obtained from sources previously indicated in this report.

Culture Conditions

Culture conditions were as previously described in Part 3 of this report. Hepatocytes were plated at a density of 7 X 10^5/chamber of 2-chamber permanox slide (Baxter Scientific Products, McGraw Park, IL). Viability of the isolated cells was at least 95% as determined by trypan blue exclusion.

Assay of Intercellular Communication

Intercellular communication between trout hepatocytes was detected by microinjection of fluorescent Lucifer Yellow CH dye into one "donor" hepatocyte and observation of dye spread (dye-coupling) to directly adjacent "recipient" hepatocytes. Microinjection was conducted using a Narishige micromanipulator (Narishige Scientific Instrument Laboratory, Tokyo, Japan) and an Eppendorf model 5242 microinjector (Eppendorf Geratebau Netheler,

Hamburg, Germany). Needles were pulled from Microdot capillaries (Glass Company of America, Millville, NJ) to a final tip diameter of approximately 0.5 uM on a Sutter Instruments P-80 Brown-Flaming micropipette puller (Sutter Instrument Company, San Rafael, CA) and were back-loaded with approximately 0.5 ul of 5% (w/v) Lucifer Yellow CH in 0.1 M LiCl, pH 7.4. Donor hepatocytes were microinjected under phase microscopy and dye-coupling to adjacent hepatocytes was evaluated 5 min later. Cultures were observed at 20°C under a Zeiss IM-35 microscope (Carl Zeiss, Inc., Thornwood, NY) at (32 X 10) X magnification. A minimum of 10 donor cells were evaluated per culture from triplicate cultures (unless otherwise stated) at each concentration and sampling time (Klaunig et al, 1989). Hepatocytes were photographed with TMAX 400 film (Eastman Kodak Co., Rochester, NY).

Dye-Coupling During Primary Culture

Hepatocytes were isolated and plated as described above. Evaluation of dye-coupling was conducted in triplicate cultures at 4, 8, 12, 24, 48 and 72 hr post plating. Cell viability was monitored in triplicate cultures at each time point by both measurement of percentage total LDH released into the medium and trypan blue exclusion. This experiment was conducted with the hepatocytes from two different trout on two separate occasions.

Effect of TPA on Dye-Coupling in 24-hr Cultures

To assess whether TPA could inhibit dye-coupling in

established cultures and if so, to determine the kinetics of inhibition, 24-hr cultures were treated with TPA (0.01, 0.10, or 1.00 ug/ml) in dimethyl sulfoxide (DMSO). These concentrations were determined to be nontoxic in preliminary dose-response experiments (Table 28). Control and vehicle control cultures were treated with 0.0% or 0.1% DMSO, respectively. Triplicate cultures were evaluated for dye-coupling at 1, 2, 3, and 4 hr post treatment. Hepatocyte viability was monitored in triplicate cultures by measurement of percentage total LDH released into the medium at each time point (Table 29). This experiment was performed in duplicate with hepatocytes from two different trout.

Based on the results obtained above, an attempt to characterize the kinetics of recovery of dye-coupling following acute exposure to TPA was also undertaken. 24-hr cultures of trout hepatocytes were exposed for 3 hr to medium containing 0.1 ug TPA/ml in DMSO or medium containing 0.1% DMSO, at which time the treatment medium was replaced with untreated medium. Although cytotoxicity as measured by percentage total LDH released (Table 29) did not indicate a reduction in cell viability in the 1.0 ug TPA/ml cultures, blebbing was observed at this concentration. As a result, the next highest concentration of TPA was chosen to avoid any confounding factors in interpratation of results. The 3-hr exposure time reflects the time required for maximum inhibition as determined in the previous experiment. Dye-coupling was recorded at 0, 1, 2, 3, and 4 hr following removal of treatment in duplicate cultures. Hepatocyte viability was monitored in duplicate cultures

by measurement of percentage total LDH released into the medium at each time point (Table 29). This experiment was conducted in duplicate with hepatocytes from two different trout.

Statistical Analysis

The statistical package STATISTIX (Version 3.5, Analytical Software, St. Paul, MN) was used for data analysis. Differences in the ratio of dye-coupled/uncoupled recipient cells between TPA-treated cultures and untreated and DMSO-treated controls were statistically analyzed with the 2 X 2 Chi-square test (Klaunig et al., 1989) (Appendix A). Although Yate's correction for 2 X 2 chi square tables has been used in the past, more recent investigators have questioned its use on the basis that it too often results in an overly conservative test (Daniel, 1974). As a result, statistical significance was based on Pearson's chi square analysis and not Yate's corrected chi square (Appendix A). The effect of "day" on dye-coupling during primary culture and treatment effects on cell viability were evaluated by one-way ANOVA.

RESULTS

Dye-Coupling During Primary Culture

Data are presented in Table 29, Figure 4, and Appendix A. Dye-coupling was detected in untreated hepatocytes 4 hours after plating. A maximum level was reached between 8 and 12 hours which was maintained up to 24 hours. Dye-coupling then decreased over

the next 48 hours. Cell viability, monitored by % total LDH released and trypan blue exclusion, began to decrease at 48 hours (Table 29). No statistically significant "day" effect was observed ($F = 0.59$; $p = 0.5218$).

Effect of TPA on Dye-Coupling in 24-Hr Cultures

Data are presented in Table 30, Figure 5, and Appendix A. No significant difference in the frequency of dye-coupled recipients was detected between nontreated and DMSO-treated cultures at any sampling time (Appendix A). 1.00 ug TPA/ml demonstrated a statistically significant reduction in dye-coupling compared to controls at 3 hours ($p = 0.0090$). Cell viability, monitored by percentage total LDH released, showed no statistically significant treatment (ANOVA results: $F = 1.84$, $p = 0.1435$) effect over all time periods (Table 31).

Effect of TPA Removal on Dye-Coupling in 24-Hr Cultures

Preliminary data are presented in Table 32, Figure 6, and Appendix A. 0.1 ug TPA/ml did not show a statistically significant inhibition of dye-coupling compared to the 0.1% DMSO control at any time period (1 hour, $p = 0.8382$; 2 hrs, $p = 0.2149$; 3 hrs, $p = 0.1619$; 4 hrs, $p = 0.0906$; Appendix A). As a result, the effect of TPA removal on dye-coupling could not be determined. Cell viability was monitored by % total LDH released (Table 33).

Table 28: Results of preliminary dose-range finding study for TPA in primary cultures of rainbow trout hepatocytes.

Treatment	% Viability[1]			
	4hr	8hr	24hr	48hr
Control	90	90	90	85
DMSO Control	93	95	93	90
1 ug TPA/ml	95	90	80	50
5 ug TPA/ml	90	80	80	50
10 ug TPA/ml	85	80	74	40
50 ug TPA/ml	75	70	60	30
100 ug TPA/ml	50	60	50	0

[1] Viability was determined by trypan blue exclusion.

Table 29: Cell viability and dye-coupling in primary cultures of female yearling rainbow trout hepatocytes over the first 72 hours in culture.

Time Post Plating (Hours)	% Total LDH Released[a]	% Viable Cells[b] (Trypan Blue Exclusion)	Dye Coupling[c]
4	6.4 ± 0.3	92 ± 1.0	39/41 (49)
8	7.6 ± 0.6	92 ± 1.0	50/40 (57)
12	5.8 ± 0.5	90 ± 0.7	65/42 (60)
24	6.2 ± 0.7	88 ± 1.6	50/38 (57)
48	8.9 ± 0.9	84 ± 1.6	32/57 (36)
72	15.9 ± 0.8	79 ± 1.3	34/54 (38)

[a] Values represent the mean ± SE of the percentage of total LDH released; n = 6 cultures, 3 cultures from each of two trout.
[b] Values represent the mean ± SE of the percentage of cells not stained with trypan blue; n = 6 cultures, 3 cultures from each of two trout.
[c] Values represent the number of coupled/noncoupled cell recipients; ( ) = the percentage of total recipient cells dye coupled; n = 6 cultures, 3 cultures from each of two trout. A minimum of 10 donor cells per culture were evaluated at each concentration and sampling time (Klaunig et al., 1989).

Note: The experiments (1 trout/experiment) were conducted on two different days. No statistically significant day effect was observed ($F = 0.59$, $p = 0.5128$)

Table 30: Dye-coupling[a] in 24-hour primary cultures of female yearling rainbow trout hepatocytes following treatment with TPA.

Treatment	Duration of Exposure (Hours) 1	2	3	4
Control	64/48 (56%)	64/46 (58)	63/45 (58)	62/47 (57)
0.1% DMSO	54/51 (52)	60/45 (57)	56/50 (53)	61/50 (55)
TPA ug/ml				
0.01	58/50 (54)	52/48 (52)	54/50 (52)	54/51 (51)
0.10	50/50 (50)	49/52 (49)	44/58 (43)	47/61 (43)
1.00	52/55 (49)	45/60 (43)	42/62* (40)	43/62 (41)

[a] Values represent the number of coupled/noncoupled cell recipients; ( ) = the percentage of total recipient cells dye-coupled.
* Values that are statistically different ($p < 0.0125$; Bonferroni correction for multiple comparisons of doses and vehicle control verses control at a single time point is $\alpha(0.05)/4$) from respective control cultures as determined by 2 X 2 Chi-squared analysis.
Note: The experiments (1 trout/experiment) were conducted on two different days; n = 6 cultures, 3 cultures from each of two trout. A minimum of 10 donor cells per culture were evaluated at each concentration and sampling time (Klaunig et al., 1989).

Table 31: Percentage of total LDH released[1] in 24-hour primary cultures of female yearling rainbow trout hepatocytes following treatment with TPA.

Treatment	Duration of Exposure (Hours)			
	1	2	3	4
Control	6.0 ± 0.6	6.1 ± 0.3	5.3 ± 0.3	6.2 ± 0.3
0.1% DMSO	6.2 ± 0.5	6.6 ± 1.1	6.4 ± 1.3	6.5 ± 0.8
0.01 ug TPA/ml	6.3 ± 0.1	6.2 ± 0.1	6.2 ± 0.8	6.5 ± 0.6
0.1 ug TPA/ml	6.1 ± 0.1	7.5 ± 0.8	6.3 ± 0.2	6.2 ± 0.1
1.0 ug TPA/ml	7.0 ± 1.2	6.9 ± 0.2	6.9 ± 0.2	6.6 ± 0.4

[1] Values represent the mean ± SE of the percentage of total LDH released; n = 2 cultures, 1 culture from each of two trout.

Table 32: Dye-coupling[1] in 24-hour primary cultures of female yearling rainbow trout hepatocytes following removal of TPA[2].

	Recovery Time (Hours)				
Treatment	0	1	2	3	4
0.1% DMSO	39/32 (55)	35/33 (53)	36/33 (52)	37/33 (53)	36/36 (50)
0.1 ug TPA/ml	32/39 (45)	33/38 (45)	35/39 (47)	34/35 (47)	30/37 (43)

[1] Values represent the number of coupled/noncoupled cell recipients; ( ) = the percentage of total recipient cells dye-coupled; n = 4 cultures, 2 cultures from each of two trout. A minimum of 10 donor cells per culture were evaluated at each concentration and sampling time (Klaunig et al., 1989).
[2] Cells were exposed to 0.1 ug TPA/ml media for 3 hours prior to removal.

Table 33: Percentage of total LDH released[1] in 24-hour primary cultures of female yearling rainbow trout hepatocytes following removal of TPA[2].

	Recovery Time (Hours)			
Treatment	1	2	3	4
0.1% DMSO	5.1 ± 0.5	4.9 ± 0.8	5.4 ± 0.6	7.8 ± 0.3
0.1 ug TPA/ml	4.2 ± 0.7	4.1 ± 0.5	4.8 ± 0.8	6.0 ± 0.6

[1] Values represent the mean ± SE of the percentage of total LDH released; n = 2 cultures, 1 culture from each of two trout.
[2] Cells were exposed to 0.1 ug TPA/ml media for 3 hours prior to removal.

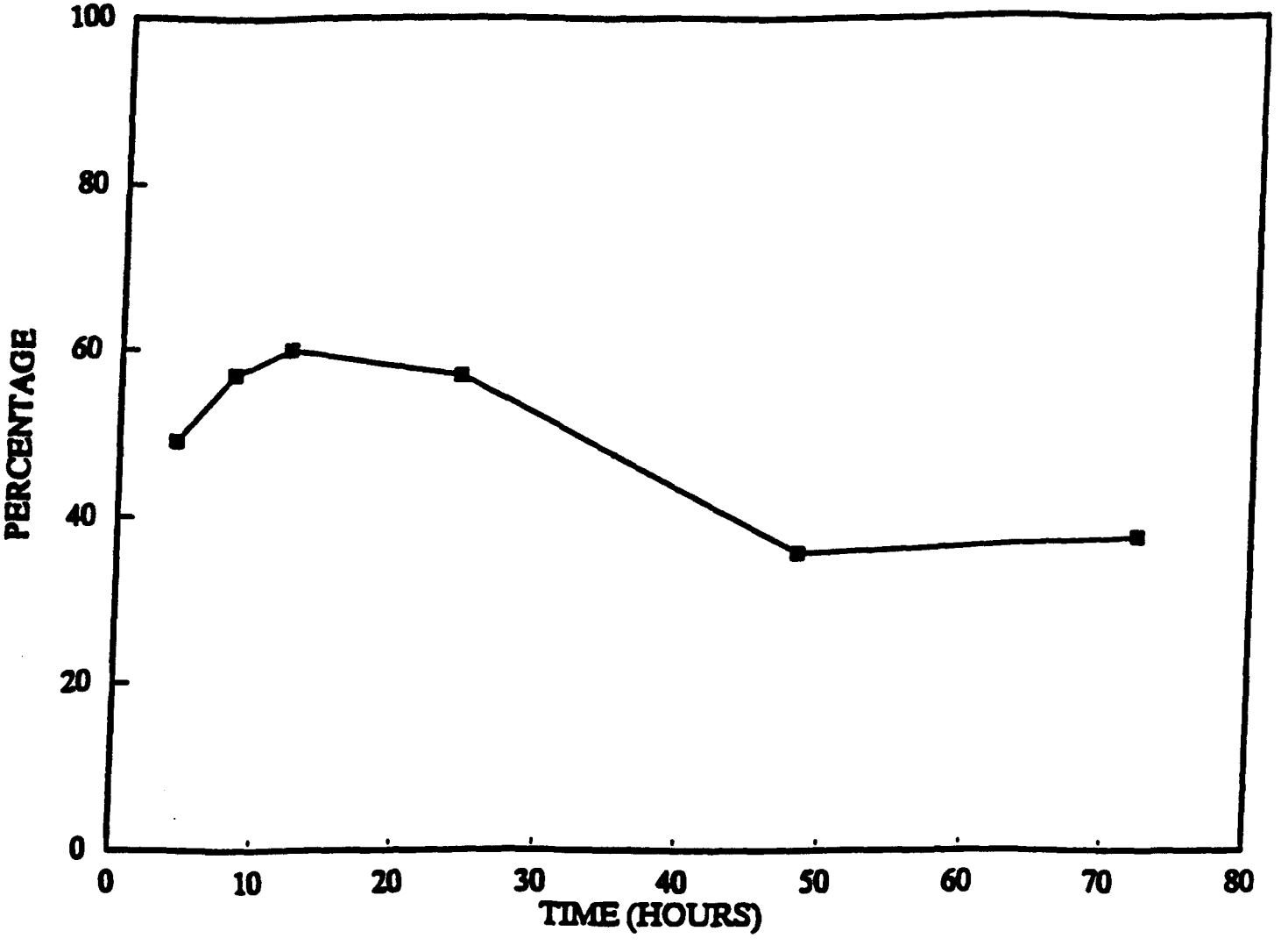

Figure 4. Percentage of dye-coupled recipient cells over time in primary cultures of untreated rainbow trout hepatocytes.

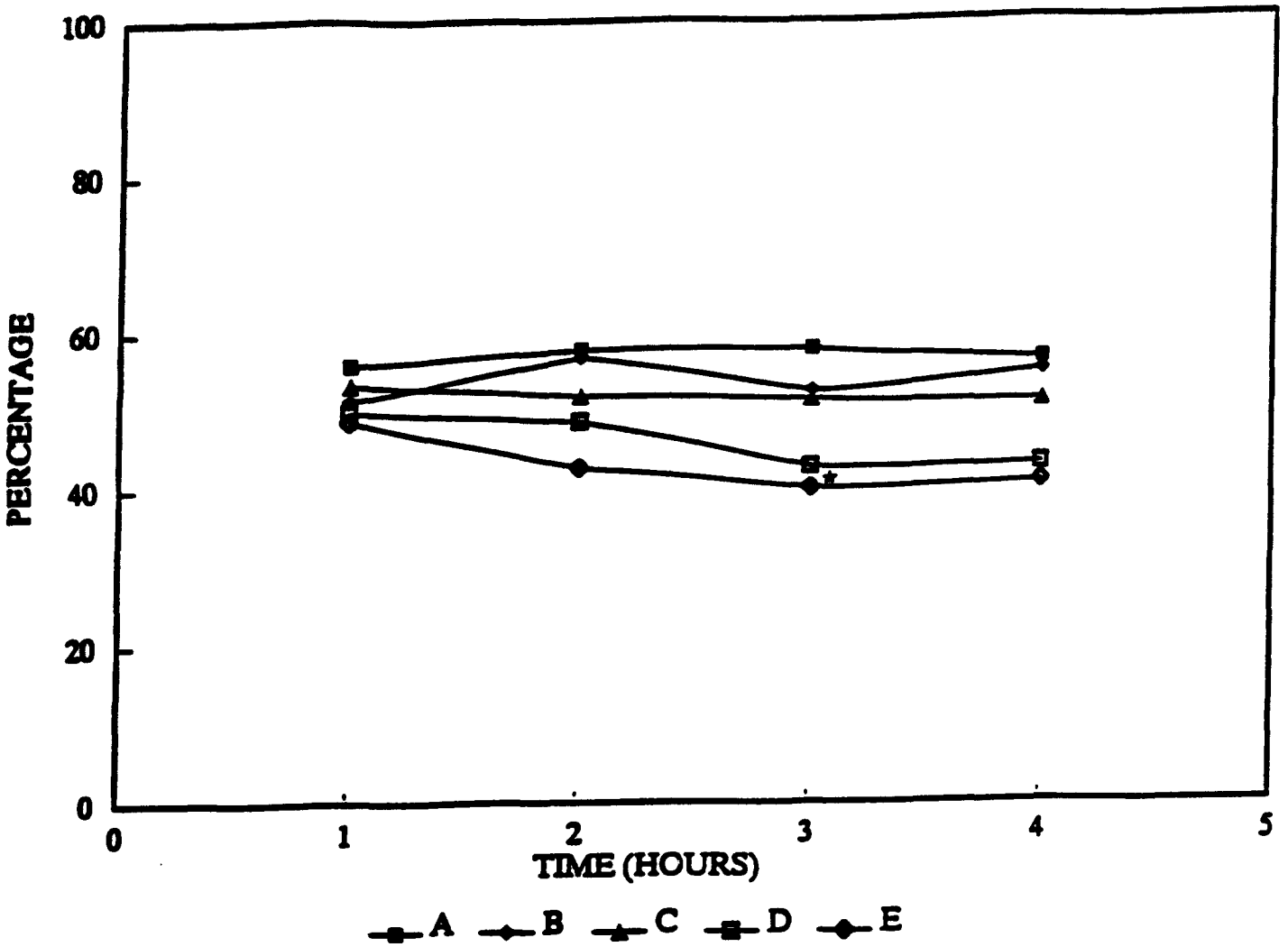

Figure 5. Dose-related inhibition of dye-coupled recipient cells in 24-hour cultures of rainbow trout hepatocytes treated with TPA: A = untreated control; B = 0.1% DMSO control; C = 0.01 ug TPA/ml; D = 0.10 ug TPA/ml; and E = 1.00 ug TPA/ml (statistically significant ($p < 0.0125$) from respective control (*).

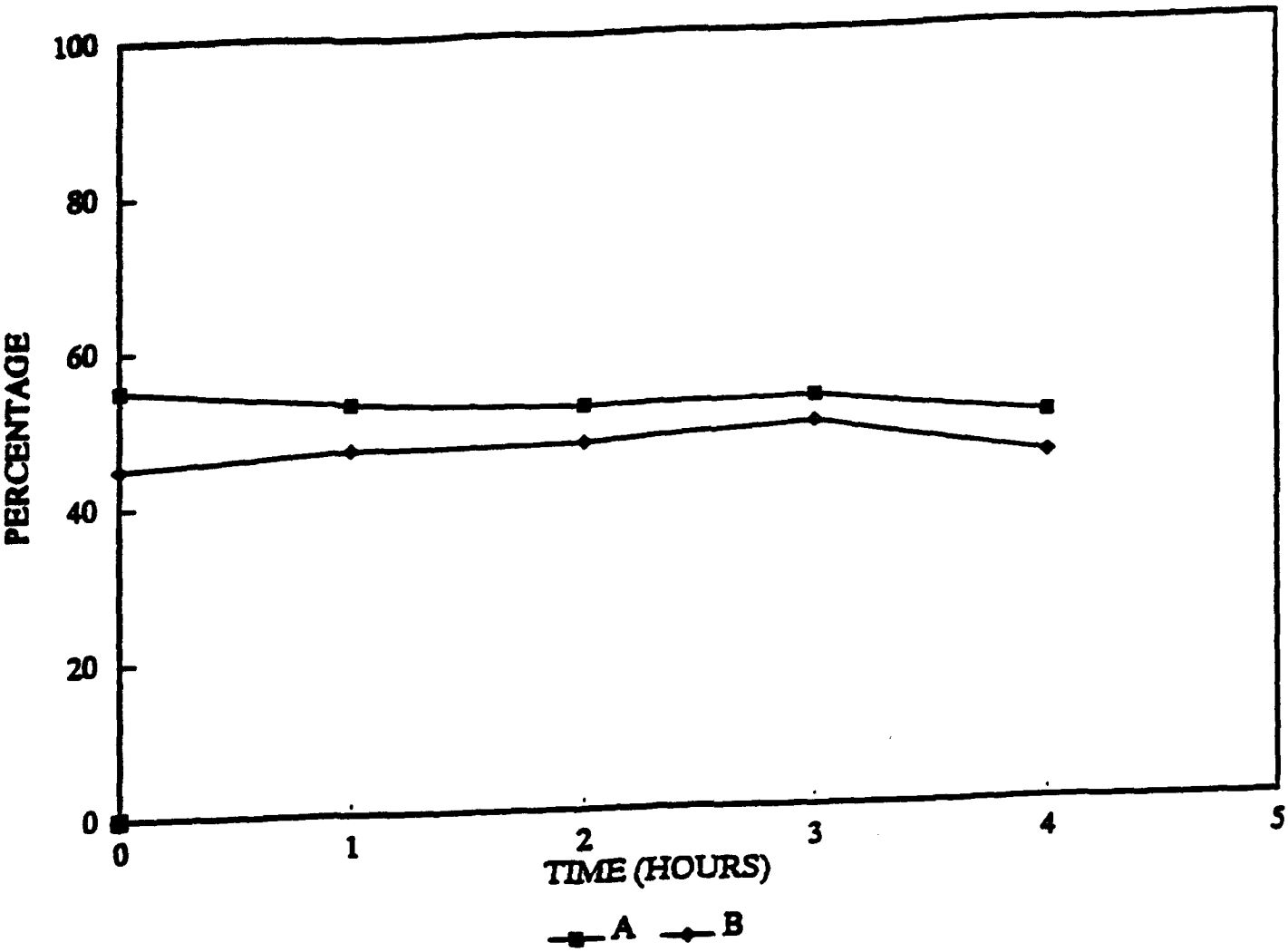

Figure 6. Dye-coupling in 24-hour primary cultures of rainbow trout hepatocytes following removal of TPA. Cells were exposed to 0.1 ug TPA/ml media for 3 hours prior to removal; A = 0.1% DMSO control; B = 0.1 ug TPA/ml media.

DISCUSSION

This study was designed to determine the extent of intercellular communication (dye-coupling) in nontreated primary cultures of rainbow trout hepatocytes and to evaluate this system as a potential *in vitro* model for dye-coupling experiments. The results showed that dye-coupling increased during the first 8 hours in culture, was maintained up to 24 hours, and then decreased over the next 48 hours. The maximum percentage of dye-coupled recipients reached was 60%. This is lower compared to similar studies with primary cultures of rodent hepatocytes where maximum levels of dye-coupling were approximately 90% (Klaunig et al., 1989).

Preliminary studies were conducted with 24-hour cultures to test the effect of TPA, a known inhibitor of rodent intercellular communication, on established, functioning trout gap junctions. The data revealed a dose-related inhibition of dye-coupled recipient cells although statistically significant differences compared to the control were observed only at the highest concentration of TPA at 3 hours. As a result, the attempt to document the kinetics of recovery from TPA-induced inhibition of dye-coupling was not successful since 0.1 ug TPA/ml did not show a statistically significant inhibition of dye-coupling compared to the 0.1% DMSO control at any time period.

Compared to *in vitro* rodent models, the present rainbow trout model does not exhibit a high level of dye-coupling. Although the standard serum-free culture conditions used in this study were

designed to promote attachment, viability and function (Donohue et al., 1993), further efforts should be directed towards optimizing the conditions of the culture system to facilitate the dye-coupling response. Nonetheless, this preliminary data demonstrate the potential of this in vitro model system for the study of the effects of agents on intercellular communication and provide a basis for future research.

CONCLUSIONS

1. Intercellular communication is established in untreated hepatocytes 4 hours after plating.

2. A maximum level is reached between 8 and 12 hours which is maintained up to 24 hours. Dye-coupling then decreases over the next 48 hours.

3. TPA inhibits dye-coupling in 24 hour cultures in a dose-dependent manner. A maximum level of inhibition is reached at 3 hours of exposure.

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PART 7: PROJECT SUMMARY

GENERAL

The objective of this project was to assess whether fish used as toxicological models had the potential to display biochemical changes associated with epigenetic mechanisms for carcinogenesis. The research direction was viewed as complementary to the principal direction of environmental carcinogenesis research which has focused on genotoxic agents and their respective mechanisms of action. The project sought, therefore, to assess whether the fish model(s) would display the capacity for peroxisome proliferation, chemically-induced mitogenicity, hepatic ornithine decarboxylase activity and cell-to-cell communication.

Since none of these techniques had been successfully applied to the fish models proposed for use in the project, considerable effort was devoted to the development of the respective assays and techniques and refining each for the particular fish in vivo and in vitro model. In addition, much effort was devoted to develop micro-assays in order to improve upon previous research efforts with medaka in which pooling of numerous medaka were necessary for endpoint measurement. Our goal in all such cases was to achieve reproducible values on single medaka.

SPECIFIC

PEROXISOME PROLIFERATION

The overall findings indicate that rainbow trout and medaka respond to treatment with peroxisome proliferators as measured by increases in fatty acyl-CoA oxidase (FACO), and the peroxisome bifunctional enzyme (PBE), as well as in the increase of volume density of peroxisomes in the case of trout. The fish models generally responded in a qualitatively similar manner to peroxisome proliferation as seen with rodents. That is, those peroxisome proliferaters (e.g., ciprofibrate and clofibrate) that are the most potent in rodents are the most potent in fish. However, in a quantitative sense, the fish were considerably less sensitive than the rodent with respect to both background peroxisomal enzyme activity and the capacity for their induction.

In completing the peroxisome proliferation portion of the project, the research team identified for the first time the peroxisomal bi-functional enzyme (PBE) associated with peroxisome proliferation and the adoption and refinement of an enzyme assay for FACO (i.e., Small et al., 1985) that is some 50-fold more sensitive than Lazaro's assay, that was employed at the start of the project. In addition, the project developed an <u>in vitro</u> system to assess the potential for peroxisome proliferation. These were developed for both the rainbow trout and medaka. The findings generally confirmed <u>in vivo</u> responses and offered exciting potential as future model systems for multiple endpoint

including, but not restricted to, peroxisome proliferation.

MITOGENICITY

As in the case of peroxisome proliferation new developmental techniques and procedures were required in order to assess mitogenic potential of environmental agents. However, once techniques were developed and refined they were applied to assessing the mitogenic potential of peroxisome proliferating agents as well as a positive control (i.e., inorganic lead salts).

The experimentation revealed that the peroxisomal proliferating agents displayed relatively low capacity to induce mitogenic responses as measured by uptake of tritiated thymidine.

A large *in vivo* study exposing rainbow trout to inorganic lead revealed no significant treatment effects based on liver to body weight ratio and total hepatic DNA content. Similar experiments with rats resulted in profound increases in the liver to body weight ratio and in the hepatic content of DNA. Lead is clearly a strong mitogen for the rat, but not for the rainbow trout fish model.

HEPATIC ORNITHINE DECARBOXYLASE

Considerable efforts were made to develop a reproducible and quantitative hepatic ODC assay. Once developed, the ODC assay

was applied to male and female medaka and trout. Of greatest interest was that the ODC activity was 100-200-fold higher in the fish models than rodents. These findings provided the first major promotional parameter whereby fish displayed a pronounced increase over rodents. Of further interest is that subsequent research has shown that the ODC activity levels could be manipulated via the use of the mammalian ODC inhibitor DMFO.

CELL-TO-CELL COMMUNICATION

The project provided the first reported study of cell-to-cell communication in fish toxicological models in vitro (Final Report). Baseline cell-to-cell communication values were established and compared with published mammalian findings. The experimental system was then challenged with the well known tumor promoter TPA which adversely affected cell-to-cell communication in fish hepatocytes.

OVERALL

This project achieved strong success in the development of new experimental approaches and methods for assessing epigenetic carcinogenesis in fish models. In four instances (i.e., identification of PBE, quantifying uptake of tritiated thymidine in primary hepatocytes, development of an hepatic ODC assay, and documenting cell-to-cell communication) the project provided the cutting edge research on the application of these techniques to

fish toxicology. Similarly important is that the research on ODC provided values on individual medaka so that interindividual variation could be assessed.

The findings indicate that rainbow trout and medaka were weak responders with respect to peroxisome proliferating agents and in our mitogenicity system. However, their extremely high hepatic ODC activity represents an exciting area of follow-up research with respect to tumor promotion and progression.

APPENDIX A:

2 X 2 Chi Square Analysis of Part 6 Dye-Coupling Data

Dye-coupling in 24 hour primary cultures of female rainbow trout hepatocytes following treatment with TPA (Table 30).

Control vs. 0.1% DMSO at 1 hour

64	48
54	51

PEARSON'S CHI SQUARE	0.71	YULE'S Q	0.11
P (PEARSON'S)	0.3983	SE (Q)	1.348E-01
YATES' CORRECTED CHI SQ	0.50	SE (H0: Q = 0)	1.364E-01
P (YATES)	0.4788	YULE'S Y	0.06
LOG ODDS RATIO	2.305E-01	SE (Y)	6.805E-02
SE (LOR)	2.731E-01	SE (H0: Y = 0)	6.818E-02
SE (H0: LOR = 0)	2.727E-01	C MAX	0.69
CROSS PRODUCT RATIO	1.259	PHI	0.06
CONTINGENCY COEFF	0.06	PHI MAX	0.95

Control vs. 0.1% DMSO at 2 hours

64	46
60	45

PEARSON'S CHI SQUARE	0.02	YULE'S Q	0.02
P (PEARSON'S)	0.8775	SE (Q)	1.380E-01
YATES' CORRECTED CHI SQ	0.00	SE (H0: Q = 0)	1.381E-01
P (YATES)	0.9872	YULE'S Y	0.01
LOG ODDS RATIO	4.256E-02	SE (Y)	6.903E-02
SE (LOR)	2.761E-01	SE (H0: Y = 0)	6.904E-02
SE (H0: LOR = 0)	2.761E-01	C MAX	0.69
CROSS PRODUCT RATIO	1.043	PHI	0.01
CONTINGENCY COEFF	0.01	PHI MAX	0.88

Control vs. 0.1% DMSO at 3 hours

63	45
56	50

PEARSON'S CHI SQUARE	0.66	YULE'S Q	0.11
P (PEARSON'S)	0.4179	SE (Q)	1.361E-01
YATES' CORRECTED CHI SQ	0.45	SE (H0: Q = 0)	1.376E-01
P (YATES)	0.5012	YULE'S Y	0.06
LOG ODDS RATIO	2.231E-01	SE (Y)	6.868E-02
SE (LOR)	2.756E-01	SE (H0: Y = 0)	6.880E-02
SE (H0: LOR = 0)	2.752E-01	C MAX	0.67
CROSS PRODUCT RATIO	1.250	PHI	0.06
CONTINGENCY COEFF	0.06	PHI MAX	0.90

Control vs. 0.1% DMSO at 4 hours

62	47
61	50

PEARSON'S CHI SQUARE	0.08	YULE'S Q	0.04
P (PEARSON'S)	0.7736	SE (Q)	1.356E-01
YATES' CORRECTED CHI SQ	0.02	SE (H0: Q = 0)	1.356E-01
P (YATES)	0.8793	YULE'S Y	0.02
LOG ODDS RATIO	7.814E-02	SE (Y)	6.789E-02
SE (LOR)	2.717E-01	SE (H0: Y = 0)	6.790E-02
SE (H0: LOR = 0)	2.716E-01	C MAX	0.66
CROSS PRODUCT RATIO	1.081	PHI	0.02
CONTINGENCY COEFF	0.02	PHI MAX	0.88

Control vs. 0.01 ug TPA/ml at 1 hour

64	48
58	50

PEARSON'S CHI SQUARE	0.26	YULE'S Q	0.07
P (PEARSON'S)	0.6079	SE (Q)	1.351E-01
YATES' CORRECTED CHI SQ	0.14	SE (H0: Q = 0)	1.357E-01
P (YATES)	0.7059	YULE'S Y	0.03
LOG ODDS RATIO	1.393E-01	SE (Y)	6.779E-02
SE (LOR)	2.715E-01	SE (H0: Y = 0)	6.784E-02
SE (H0: LOR = 0)	2.713E-01	C MAX	0.67
CROSS PRODUCT RATIO	1.149	PHI	0.03
CONTINGENCY COEFF	0.03	PHI MAX	0.91

Control vs. 0.01 ug TPA/ml at 2 hours

64	46
52	48

PEARSON'S CHI SQUARE	0.81	YULE'S Q	0.12
P (PEARSON'S)	0.3682	SE (Q)	1.370E-01
YATES' CORRECTED CHI SQ	0.58	SE (H0: Q = 0)	1.389E-01
P (YATES)	0.4468	YULE'S Y	0.06
LOG ODDS RATIO	2.502E-01	SE (Y)	6.929E-02
SE (LOR)	2.783E-01	SE (H0: Y = 0)	6.947E-02
SE (H0: LOR = 0)	2.779E-01	C MAX	0.69
CROSS PRODUCT RATIO	1.284	PHI	0.06
CONTINGENCY COEFF	0.06	PHI MAX	0.94

Control vs. 0.01 ug TPA/ml at 3 hours

63	45
54	50

PEARSON'S CHI SQUARE	0.88	YULE'S Q	0.13
P (PEARSON'S)	0.3481	SE (Q)	1.361E-01
YATES' CORRECTED CHI SQ	0.64	SE (H0: Q = 0)	1.381E-01
P (YATES)	0.4236	YULE'S Y	0.06
LOG ODDS RATIO	2.595E-01	SE (Y)	6.891E-02
SE (LOR)	2.768E-01	SE (H0: Y = 0)	6.907E-02
SE (H0: LOR = 0)	2.763E-01	C MAX	0.68
CROSS PRODUCT RATIO	1.296	PHI	0.06
CONTINGENCY COEFF	0.06	PHI MAX	0.92

Control vs. 0.01 ug TPA/ml at 4 hours

62	47
54	51

PEARSON'S CHI SQUARE	0.64	YULE'S Q	0.11
P (PEARSON'S)	0.4235	SE (Q)	1.358E-01
YATES' CORRECTED CHI SQ	0.44	SE (H0: Q = 0	1.372E-01
P (YATES)	0.5073	YULE'S Y	0.05
LOG ODDS RATIO	2.198E-01	SE (Y)	6.850E-02
SE (LOR)	2.749E-01	SE (H0: Y = 0	6.861E-02
SE (H0: LOR = 0)	2.745E-01	C MAX	0.68
CROSS PRODUCT RATIO	1.246	PHI	0.05
CONTINGENCY COEFF	0.05	PHI MAX	0.94

Control vs. 0.1 ug TPA/ml at 1 hour

64	48
50	50

PEARSON'S CHI SQUARE	1.08	YULE'S Q	0.14
P (PEARSON'S)	0.2977	SE (Q)	1.354E-01
YATES' CORRECTED CHI SQ	0.82	SE (H0: Q = 0)	1.380E-01
P (YATES)	0.3663	YULE'S Y	0.07
LOG ODDS RATIO	2.877E-01	SE (Y)	6.877E-02
SE (LOR)	2.765E-01	SE (H0: Y = 0)	6.899E-02
SE (H0: LOR = 0)	2.759E-01	C MAX	0.70
CROSS PRODUCT RATIO	1.333	PHI	0.07
CONTINGENCY COEFF	0.07	PHI MAX	0.98

Control vs. 0.1 ug TPA/ml at 2 hours

64	46
49	52

PEARSON'S CHI SQUARE	1.98	YULE'S Q	0.19
P (PEARSON'S)	0.1596	SE (Q)	1.336E-01
YATES' CORRECTED CHI SQ	1.61	SE (H0: Q = 0)	1.382E-01
P (YATES)	0.2047	YULE'S Y	0.10
LOG ODDS RATIO	3.897E-01	SE (Y)	6.872E-02
SE (LOR)	2.775E-01	SE (H0: Y = 0)	6.908E-02
SE (H0: LOR = 0)	2.763E-01	C MAX	0.70
CROSS PRODUCT RATIO	1.476	PHI	0.10
CONTINGENCY COEFF	0.10	PHI MAX	0.97

Control vs. 0.1 ug TPA/ml at 3 hours

63	45
44	58

PEARSON'S CHI SQUARE	4.85	YULE'S Q	0.30
P (PEARSON'S)	0.0277	SE (Q)	1.274E-01
YATES' CORRECTED CHI SQ	4.26	SE (H0: Q = 0)	1.381E-01
P (YATES)	0.0391	YULE'S Y	0.15
LOG ODDS RATIO	6.127E-01	SE (Y)	6.824E-02
SE (LOR)	2.794E-01	SE (H0: Y = 0)	6.905E-02
SE (H0: LOR = 0)	2.762E-01	C MAX	0.70
CROSS PRODUCT RATIO	1.845	PHI	0.15
CONTINGENCY COEFF	0.15	PHI MAX	0.99

Control vs. 0.1 ug TPA/ml at 4 hours

62	47
47	61

PEARSON'S CHI SQUARE	3.87	YULE'S Q	0.26
P (PEARSON'S)	0.0490	SE (Q)	1.276E-01
YATES' CORRECTED CHI SQ	3.36	SE (H0: Q = 0)	1.358E-01
P (YATES)	0.0669	YULE'S Y	0.13
LOG ODDS RATIO	5.377E-01	SE (Y)	6.728E-02
SE (LOR)	2.740E-01	SE (H0: Y = 0)	6.789E-02
SE (H0: LOR = 0)	2.715E-01	C MAX	0.71
CROSS PRODUCT RATIO	1.712	PHI	0.13
CONTINGENCY COEFF	0.13	PHI MAX	1.00

Control vs. 1.0 ug TPA/ml at 1 hour

64	48
52	55

PEARSON'S CHI SQUARE	1.60	YULE'S Q	0.17
P (PEARSON'S)	0.2054	SE (Q)	1.320E-01
YATES' CORRECTED CHI SQ	1.28	SE (H0: Q = 0)	1.354E-01
P (YATES)	0.2581	YULE'S Y	0.09
LOG ODDS RATIO	3.438E-01	SE (Y)	6.745E-02
SE (LOR)	2.718E-01	SE (H0: Y = 0)	6.771E-02
SE (H0: LOR = 0)	2.708E-01	C MAX	0.69
CROSS PRODUCT RATIO	1.410	PHI	0.09
CONTINGENCY COEFF	0.09	PHI MAX	0.96

Control vs. 1.0 ug TPA/ml at 2 hours

64	46
45	50

PEARSON'S CHI SQUARE	5.05	YULE'S Q	0.30
P (PEARSON'S)	0.0247	SE (Q)	1.257E-01
YATES' CORRECTED CHI SQ	4.45	SE (H0: Q = 0)	1.364E-01
P (YATES)	0.0348	YULE'S Y	0.15
LOG ODDS RATIO	6.179E-01	SE (Y)	6.741E-02
SE (LOR)	2.761E-01	SE (H0: Y = 0)	6.822E-02
SE (H0: LOR = 0)	2.729E-01	C MAX	0.70
CROSS PRODUCT RATIO	1.855	PHI	0.15
CONTINGENCY COEFF	0.15	PHI MAX	0.99

Control vs. 1.0 ug TPA/ml at 3 hours

63	45
42	62

PEARSON'S CHI SQUARE	6.83	YULE'S Q	0.35
P (PEARSON'S)	0.0090	SE (Q)	1.228E-01
YATES' CORRECTED CHI SQ	6.13	SE (H0: Q = 0)	1.374E-01
P (YATES)	0.0133	YULE'S Y	0.18
LOG ODDS RATIO	7.259E-01	SE (Y)	6.759E-02
SE (LOR)	2.793E-01	SE (H0: Y = 0)	6.870E-02
SE (H0: LOR = 0)	2.748E-01	C MAX	0.70
CROSS PRODUCT RATIO	2.067	PHI	0.18
CONTINGENCY COEFF	0.18	PHI MAX	0.97

Control vs. 1,0 ug TPA/ml at 4 hours

62	47
43	62

PEARSON'S CHI SQUARE	5.43	YULE'S Q	0.31
P (PEARSON'S)	0.0198	SE (Q)	1.252E-01
YATES' CORRECTED CHI SQ	4.81	SE (H0: Q = 0)	1.368E-01
P (YATES)	0.0283	YULE'S Y	0.16
LOG ODDS RATIO	6.429E-01	SE (Y)	6.752E-02
SE (LOR)	2.771E-01	SE (H0: Y = 0)	6.838E-02
SE (H0: LOR = 0)	2.735E-01	C MAX	0.69
CROSS PRODUCT RATIO	1.902	PHI	0.16
CONTINGENCY COEFF	0.16	PHI MAX	0.96

0.1% DMSO vs. 0.01 ug TPA/ml at 1 hour

54	51
58	50

PEARSON'S CHI SQUARE	0.11	YULE'S Q	-0.05
P (PEARSON'S)	0.7395	SE (Q)	1.370E-01
YATES' CORRECTED CHI SQ	0.04	SE (H0: Q = 0)	1.372E-01
P (YATES)	0.8452	YULE'S Y	-0.02
LOG ODDS RATIO	-9.126E-02	SE (Y)	6.860E-02
SE (LOR)	2.745E-01	SE (H0: Y = 0)	6.862E-02
SE (H0: LOR = 0)	2.745E-01	C MAX	0.69
CROSS PRODUCT RATIO	9.128E-01	PHI	-0.02
CONTINGENCY COEFF	0.02	PHI MAX	-0.96

0.1% DMSO vs. 0.01 ug TPA/ml at 2 hours

60	45
52	48

PEARSON'S CHI SQUARE	0.55	YULE'S Q	0.10
P (PEARSON'S)	0.4597	SE (Q)	1.390E-01
YATES' CORRECTED CHI SQ	0.36	SE (H0: Q = 0)	1.403E-01
P (YATES)	0.5492	YULE'S Y	0.05
LOG ODDS RATIO	2.076E-01	SE (Y)	7.006E-02
SE (LOR)	2.810E-01	SE (H0: Y = 0)	7.017E-02
SE (H0: LOR = 0)	2.807E-01	C MAX	0.68
CROSS PRODUCT RATIO	1.231	PHI	0.05
CONTINGENCY COEFF	0.05	PHI MAX	0.93

0.1% DMSO vs. 0.01 ug TPA/ml at 3 hours

56	50
54	50

PEARSON'S CHI SQUARE	0.02	YULE'S Q	0.02
P (PEARSON'S)	0.8953	SE (Q)	1.381E-01
YATES' CORRECTED CHI SQ	0.00	SE (H0: Q = 0)	1.382E-01
P (YATES)	1.0000	YULE'S Y	0.01
LOG ODDS RATIO	3.637E-02	SE (Y)	6.908E-02
SE (LOR)	2.764E-01	SE (H0: Y = 0)	6.909E-02
SE (H0: LOR = 0)	2.764E-01	C MAX	0.69
CROSS PRODUCT RATIO	1.037	PHI	0.01
CONTINGENCY COEFF	0.01	PHI MAX	0.96

0.1% DMSO vs. 0.01 ug TPA/ml at 4 hours

61	50
54	51

PEARSON'S CHI SQUARE	0.27	YULE'S Q	0.07
P (PEARSON'S)	0.6036	SE (Q)	1.358E-01
YATES' CORRECTED CHI SQ	0.15	SE (H0: Q = 0)	1.364E-01
P (YATES)	0.7019	YULE'S Y	0.04
LOG ODDS RATIO	1.417E-01	SE (Y)	6.816E-02
SE (LOR)	2.730E-01	SE (H0: Y = 0)	6.821E-02
SE (H0: LOR = 0)	2.728E-01	C MAX	0.69
CROSS PRODUCT RATIO	1.152	PHI	0.04
CONTINGENCY COEFF	0.04	PHI MAX	0.96

0.1% DMSO vs. 0.1 ug TPA/ml at 1 hour

54	51
50	50

PEARSON'S CHI SQUARE	0.04	YULE'S Q	0.03
P (PEARSON'S)	0.8380	SE (Q)	1.396E-01
YATES' CORRECTED CHI SQ	0.00	SE (H0: Q = 0)	1.397E-01
P (YATES)	0.9484	YULE'S Y	0.01
LOG ODDS RATIO	5.716E-02	SE (Y)	6.986E-02
SE (LOR)	2.795E-01	SE (H0: Y = 0)	6.987E-02
SE (H0: LOR = 0)	2.795E-01	C MAX	0.70
CROSS PRODUCT RATIO	1.059	PHI	0.01
CONTINGENCY COEFF	0.01	PHI MAX	0.99

0.1% DMSO vs. 0.1 ug TPA/ml at 2 hours

60	45
49	52

PEARSON'S CHI SQUARE	1.54	YULE'S Q	0.17
P (PEARSON'S)	0.2149	SE (Q)	1.360E-01
YATES' CORRECTED CHI SQ	1.21	SE (H0: Q = 0)	1.396E-01
P (YATES)	0.2711	YULE'S Y	0.09
LOG ODDS RATIO	3.471E-01	SE (Y)	6.953E-02
SE (LOR)	2.802E-01	SE (H0: Y = 0)	6.980E-02
SE (H0: LOR = 0)	2.792E-01	C MAX	0.69
CROSS PRODUCT RATIO	1.415	PHI	0.09
CONTINGENCY COEFF	0.09	PHI MAX	0.96

0.1% DMSO vs. 0.1 ug TPA/ml at 3 hours

56	50
44	58

PEARSON'S CHI SQUARE	1.96	YULE'S Q	0.19
P (PEARSON'S)	0.1619	SE (Q)	1.343E-01
YATES' CORRECTED CHI SQ	1.59	SE (H0: Q = 0)	1.388E-01
P (YATES)	0.2077	YULE'S Y	0.10
LOG ODDS RATIO	3.896E-01	SE (Y)	6.909E-02
SE (LOR)	2.790E-01	SE (H0: Y = 0)	6.940E-02
SE (H0: LOR = 0)	2.776E-01	C MAX	0.69
CROSS PRODUCT RATIO	1.476	PHI	0.10
CONTINGENCY COEFF	0.10	PHI MAX	0.94

0.1% DMSO vs. 0.1 ug TPA/ml at 4 hours

61	50
47	61

PEARSON'S CHI SQUARE	2.86	YULE'S Q	0.23
P (PEARSON'S)	0.0906	SE (Q)	1.291E-01
YATES' CORRECTED CHI SQ	2.43	SE (H0: Q = 0)	1.352E-01
P (YATES)	0.1194	YULE'S Y	0.11
LOG ODDS RATIO	4.596E-01	SE (Y)	6.715E-02
SE (LOR)	2.721E-01	SE (H0: Y = 0)	6.759E-02
SE (H0: LOR = 0)	2.703E-01	C MAX	0.70
CROSS PRODUCT RATIO	1.583	PHI	0.11
CONTINGENCY COEFF	0.11	PHI MAX	0.97

0.1% DMSO vs. 1.0 ug TPA/ml at 1 hour

54	51
52	55

PEARSON'S CHI SQUARE	0.17	YULE'S Q	0.06
P (PEARSON'S)	0.6803	SE (Q)	1.370E-01
YATES' CORRECTED CHI SQ	0.08	SE (H0: Q = 0)	1.374E-01
P (YATES)	0.7835	YULE'S Y	0.03
LOG ODDS RATIO	1.132E-01	SE (Y)	6.866E-02
SE (LOR)	2.748E-01	SE (H0: Y = 0)	6.868E-02
SE (H0: LOR = 0)	2.747E-01	C MAX	0.70
CROSS PRODUCT RATIO	1.120	PHI	0.03
CONTINGENCY COEFF	0.03	PHI MAX	0.99

0.1% DMSO vs. 1.0 ug TPA/ml at 2 hours

60	45
45	60

PEARSON'S CHI SQUARE	4.29	YULE'S Q	0.28
P (PEARSON'S)	0.0384	SE (Q)	1.285E-01
YATES' CORRECTED CHI SQ	3.73	SE (H0: Q = 0)	1.380E-01
P (YATES)	0.0533	YULE'S Y	0.14
LOG ODDS RATIO	5.754E-01	SE (Y)	6.830E-02
SE (LOR)	2.789E-01	SE (H0: Y = 0)	6.901E-02
SE (H0: LOR = 0)	2.760E-01	C MAX	0.71
CROSS PRODUCT RATIO	1.778	PHI	0.14
CONTINGENCY COEFF	0.14	PHI MAX	1.00

0.1% DMSO vs. 1.0 ug TPA/ml at 3 hours

56	50
42	62

PEARSON'S CHI SQUARE	3.27	YULE'S Q	0.25
P (PEARSON'S)	0.0707	SE (Q)	1.310E-01
YATES' CORRECTED CHI SQ	2.79	SE (H0: Q = 0)	1.383E-01
P (YATES)	0.0951	YULE'S Y	0.13
LOG ODDS RATIO	5.028E-01	SE (Y)	6.864E-02
SE (LOR)	2.789E-01	SE (H0: Y = 0)	6.916E-02
SE (H0: LOR = 0)	2.767E-01	C MAX	0.68
CROSS PRODUCT RATIO	1.653	PHI	0.12
CONTINGENCY COEFF	0.12	PHI MAX	0.93

0.1% DMSO vs. 1,0 ug TPA/ml at 4 hours

61	50
43	62

PEARSON'S CHI SQUARE	4.24	YULE'S Q	0.26
P (PEARSON'S)	0.0395	SE (Q)	1.272E-01
YATES' CORRECTED CHI SQ	3.70	SE (H0: Q = 0)	1.362E-01
P (YATES)	0.0546	YULE'S Y	0.14
LOG ODDS RATIO	5.648E-01	SE (Y)	6.747E-02
SE (LOR)	2.753E-01	SE (H0: Y = 0)	6.811E-02
SE (H0: LOR = 0)	2.725E-01	C MAX	0.68
CROSS PRODUCT RATIO	1.759	PHI	0.14
CONTINGENCY COEFF	0.14	PHI MAX	0.94

Dye-coupling in 24 hour primary cultures of female rainbow trout hepatocytes following removal of TPA (Table 32).

0.1% DMSO vs. 0.1 ug TPA/ml at 0 hour

39	32
32	39

PEARSON'S CHI SQUARE	1.38	YULE'S Q	0.20
P (PEARSON'S)	0.2401	SE (Q)	1.622E-01
YATES' CORRECTED CHI SQ	1.01	SE (H0: Q = 0)	1.678E-01
P (YATES)	0.3139	YULE'S Y	0.10
LOG ODDS RATIO	3.957E-01	SE (Y)	8.351E-02
SE (LOR)	3.373E-01	SE (H0: Y = 0)	8.392E-02
SE (H0: LOR = 0)	3.357E-01	C MAX	0.71
CROSS PRODUCT RATIO	1.485	PHI	0.10
CONTINGENCY COEFF	0.10	PHI MAX	1.00

0.1% DMSO vs. 0.1 ug TPA/ml at 1 hour

35	33
33	38

PEARSON'S CHI SQUARE	0.35	YULE'S Q	0.10
P (PEARSON'S)	0.5562	SE (Q)	1.682E-01
YATES' CORRECTED CHI SQ	0.18	SE (H0: Q = 0)	1.697E-01
P (YATES)	0.5754	YULE'S Y	0.05
LOG ODDS RATIO	1.999E-01	SE (Y)	8.475E-02
SE (LOR)	3.398E-01	SE (H0: Y = 0)	8.483E-02
SE (H0: LOR = 0)	3.394E-01	C MAX	0.71
CROSS PRODUCT RATIO	1.221	PHI	0.05
CONTINGENCY COEFF	0.05	PHI MAX	1.00

0.1% DMSO vs. 0.1 ug TPA/ml at 2 hours

36	33
35	39

PEARSON'S CHI SQUARE	0.34	YULE'S Q	0.10
P (PEARSON'S)	0.5600	SE (Q)	1.660E-01
YATES' CORRECTED CHI SQ	0.17	SE (H0: Q = 0)	1.674E-01
P (YATES)	0.6778	YULE'S Y	0.05
LOG ODDS RATIO	1.952E-01	SE (Y)	8.358E-02
SE (LOR)	3.351E-01	SE (H0: Y = 0)	8.368E-02
SE (H0: LOR = 0)	3.347E-01	C MAX	0.70
CROSS PRODUCT RATIO	1.216	PHI	0.05
CONTINGENCY COEFF	0.05	PHI MAX	0.97

0.1% DMSO vs. 0.1 ug TPA/ml at 3 hours

37	33
34	35

PEARSON'S CHI SQUARE	0.18	YULE'S Q	0.07
P (PEARSON'S)	0.6728	SE (Q)	1.689E-01
YATES' CORRECTED CHI SQ	0.06	SE (H0: Q = 0)	1.697E-01
P (YATES)	0.8005	YULE'S Y	0.04
LOG ODDS RATIO	1.434E-01	SE (Y)	8.479E-02
SE (LOR)	3.396E-01	SE (H0: Y = 0)	8.484E-02
SE (H0: LOR = 0)	3.394E-01	C MAX	0.70
CROSS PRODUCT RATIO	1.154	PHI	0.04
CONTINGENCY COEFF	0.04	PHI MAX	0.99

0.1% DMSO vs. 0.1 ug TPA/ml at 4 hours

36	36
30	37

PEARSON'S CHI SQUARE	0.30	YULE'S Q	0.10
P (PEARSON'S)	0.5377	SE (Q)	1.684E-01
YATES' CORRECTED CHI SQ	0.20	SE (H0: Q = 0)	1.700E-01
P (YATES)	0.6554	YULE'S Y	0.05
LOG ODDS RATIO	2.097E-01	SE (Y)	8.488E-02
SE (LOR)	3.405E-01	SE (H0: Y = 0)	8.499E-02
SE (H0: LOR = 0)	3.399E-01	C MAX	0.68
CROSS PRODUCT RATIO	1.233	PHI	0.05
CONTINGENCY COEFF	0.05	PHI MAX	0.92

APPENDIX B:

List of Publications

PUBLICATIONS

Scarano, J.L., Calabrese, E.J., Kostecki, P.T., Baldwin, L.A., and Leonard, D.A. 1993. Evaluation of a known rodent peroxisome proliferator (gemfibrozil) in two species of fish (rainbow trout: Onchorynchus mykiss and Japanese medaka: Oryzias latipes). In prep.

Baldwin, L.A., Kostecki, P.T., and Calabrese, E.J. 1993. Gap junction-mediated intercellular communication in primary cultures of rainbow trout hepatocytes. Ecotox. Environ. Safety, Submitted.

Donohue, M., Baldwin, L.A., Leonard, D.A., and Calabrese, E.J. 1993. Effect of hypolipidemic drugs gemfibrozil, ciprofibrate and clofibric acid on peroxisomal beta-oxidation in primary cultures of rainbow trout hepatocytes. Ecotox. Environ. Safety, 26:127-132.

Baldwin, L.A., Kostecki, P.T., and Calabrese, E.J. 1993. The effects of peroxisome proliferators on S-phase synthesis in primary cultures of fish hepatocytes. Ecotox. Environ. Safety, In Press.

Bell, C.E., Baldwin, L.A., Kostecki, P.T., and Calabrese, E.J. 1993. Comparative response of rainbow trout and rat to the liver

mitogen, lead. Ecotox. Environ. Safety, In Press.

Wysynski, A.M., Baldwin, L.A., Leonard, D.A., and Calabrese, E.J. 1993. The interactive potential of joint exposure to high fat diet and selected peroxisome proliferators on peroxisomal beta-oxidation in male Wistar rats. Human Exp. Toxicol., In Press.

Calabrese, E.J., Leonard, D.A., Baldwin, L.A., and Kostecki, P.T. 1993. Ornithine decarboxylase (ODC) activity in the liver of individual medaka (Oryzias latipes) of both sexes. Ecotox. Environ. Safety, 25:19-24.

Calabrese, E.J., Baldwin, L.A., Scarano, L.J., and Kostecki, P.T. 1992. Epigenetic carcinogens in fish. Reviews in Aquatic Sciences, CRC Press, 6(2):89-96.

Ochs, J.B., Baldwin, L.A., Leonard, D.A., and Calabrese, E.J. 1992. Effects of joint exposure to selected peroxisome proliferators on hepatic acyl-CoA oxidase activity in male B6C3F1 mice. Human Exp. Toxicol., 11:83-88.

Baldwin, L.A., Calabrese, E.J., Kostecki, P.T., and Yang, J.-H. 1990. Isolation of peroxisomal enoyl-CoA hydratase in rainbow trout and immunochemical identification with the bifunctional enzyme. Fish Physiol. Biochem., 8(4):347-351.

Yang, J.H., Kostecki, P.T., and Calabrese, E.J. 1990. Peroxisome proliferation as an epigenetic mechanism of carcinogenesis: relevance to aquatic toxicology. Aquatic Toxicology and Risk Assessment: Thirteenth Volume. ASTM STP 1096. W.G. Landis and W.H. van der Shalie, Eds., American Society for Testing and Materials, Philadelphia, pp. 309-327.

Yang, J.-H., Kostecki, P.T., Calabrese, E.J., and Baldwin, L.A. 1990. Induction of peroxisome proliferation in rainbow trout exposed to cirpofibrate. Toxicol. Appl. Pharmacol., 104:476-482.

APPENDIX C:

List of Poster Presentations

AWARD

Wysynski, A.M., Baldwin, L.A., Leonard, D.A., and Calabrese, E.J. 1990. Peroxisome proliferators: Omega-3 fatty acids, ethyl chlorophenoxy isobutyrate (clofibrate) and di (2-ethylhexyl) phthalate: the interactive potential. Best Poster Award, Annual Meeting of the Northeast Chapter Society of Toxicology, Sturbridge, MA.

POSTER PRESENTATIONS

Baldwin, L.A. and Calabrese, E.J. 1993. Gap junction-mediated intercellular communication in primary cultures of rainbow trout hepatocytes. Annual Meeting of the Society of Toxicology, New Orleans, LA.

Calabrese, E.J., Leonard, D.A., Baldwin, L.A., and Kostecki, P.T. 1992. Ornithine decarboxylase (ODC) activity in the liver of individual medaka (Oryzias latipes). Annual Meeting of the Society of Environmental Toxicology and Chemistry, Cincinnati, OH.

Bell, C.E., Baldwin, L.A., Kostecki, P.T., and Calabrese, E.J. 1992. Comparative response of rainbow trout and rat to the liver mitogen, lead. Annual Meeting of the Society of Environmental

Toxicology and Chemistry, Cincinnati, OH.

Baldwin, L.A., Kostecki, P.T., and Calabrese, E.J. 1992. Effect of peroxisome proliferators on S-phase synthesis in primary cultures of fish hepatocytes. Annual Meeting of the Society of Toxicology, Seattle, WA.

Donohue, M., Baldwin, L.A., Leonard, D.A., and Calabrese, E.J. 1991. Effect of hypolipidemic drugs gemfibrozil, ciprofibrate and clofibric acid on peroxisomal beta-oxidation in primary cultures of rainbow trout hepatocytes. Annual Meeting of the Society of Toxicology and Chemistry, Seattle, WA.

Baldwin, L.A., Kostecki, P.T., and Calabrese, E.J. 1991. Induction of replicative S-phase synthesis in primary cultures of rainbow trout hepatocytes exposed to peroxisome proliferators. Annual Meeting of the Society of Toxicology and Chemistry, Seattle, WA.

Scarano, L.J., Baldwin, L.A., Leonard, D.A., Kostecki, P.T., and Calabrese, E.J. 1991. Evaluation of rainbow trout as a model for epigenetic carcinogens. 1991. Annual Meeting of the American Association for Cancer Research, Houston, TX.

Donohue, M., Baldwin, L.A., Leonard, D.A., and Calabrese, E.J. 1991. The use of primary cultures of rainbow trout hepatocytes to evaluate peroxisome proliferation potential of hypolipidemic drugs. Annual Meeting of the American Association for Cancer Research, Houston, TX.

Wysynski, A.M., Baldwin, L.A., Leonard, D.A., and Calabrese, E.J. 1991. Peroxisome proliferators: Omega-3 fatty acids, ethyl chlorophenoxy isobutyrate (clofibrate) and di (2-ethylhexyl) phthalate (DEHP): the interactive potential. Annual Meeting of the Society of Toxicology, Dallas, TX.

Scarano, L.J., Baldwin, L.A., Calabrese, E.J., and Kostecki, P.T. 1990. Lack of peroxisome proliferation in Japanese medaka exposed to DEHP or 2,4-D. Annual Meeting of the Society of Toxicology and Chemistry, Arlington, VA.

Yang, J.-H., Scarano, L.J., Baldwin, L.A., Salimi, A., Calabrese, E.J., Kostecki, P.T., and Leonard, D.A. 1990. Evaluation of various peroxisome proliferators in rainbow trout (Salmo gairdneri). Annual Meeting of the Society of Environmental Toxicology and Chemistry, Arlington, VA.

Wysynski, A.M., Baldwin, L.A., Leonard, D.A., and Calabrese, E.J. 1990. Peroxisome proliferators: Omega-3 fatty acids, ethyl

chlorophenoxy isobutyrate (clofibrate) and di (2-ethylhexyl) phthalate: the interactive potential. Annual Meeting of the Northeast Chapter Society of Toxicology, Sturbridge, MA.

Yang, J.-H., Scarano, L.J., Baldwin, L.A., Salimi, A., Calabrese, E.J., Kostecki, P.T., and Leonard, D.A. 1990. Evaluation of various peroxisome proliferators in rainbow trout (Salmo gairdneri). Annual Meeting of the Society of Toxicology, Miami Beach, FL.

Scarano, L.J., Baldwin, L.A., Leonard, D.A., Calabrese, E.J., and Kostecki, P.T. 1989. The evaluation of three peroxisome proliferators (gemfibrozil, 2,4-D and trichloroethylene) in rainbow trout. Annual Meeting of the Northeast Chapter Society of Toxicology, Sturbridge, MA.

Ochs, J., Calabrese, E.J., Kostecki, P.T., Baldwin, L.A., and Leonard, D.A. 1989. Chemical interaction potential of two peroxisome proliferators, clofibrate and ciprofibrate, in mice. Annual Meeting of the Northeast Chapter Society of Toxicology, Sturbridge, MA.

Yang, J.-H., Salimi, A., Calabrese, E.J., and Kostecki, P.T. 1988. Evaluation of the peroxisome proliferation potential of clofibrate and ciprofibrate in rainbow trout. Annual Meeting of the Northeast Chapter Society of Toxicology, Sturbridge, MA.

APPENDIX D:

List of Thesis Projects

THESIS PROJECTS

Doyle, J.M. 1993. Peroxisome proliferation potential of hypolipidemic drugs in primary cultures of medaka hepatocytes. Masters Thesis.

Scarano, J.L. 1991. Evaluation of the peroxisome proliferation potential of various compounds in two species of fish: rainbow trout (Salmo gairdneri) and Japanese medaka (Oryzias latipes). Ph.D. Thesis.

Donohue, M. 1991. The use of primary cultures of rainbow trout hepatocytes to evaluate peroxisome proliferation potential. Masters Thesis.

Wysynski, A.M. 1991. Peroxisome proliferators: Omega-3 fat ty acids, ethyl chlorophenoxyisobutyrate (clofibrate) and di (2-ethylhexyl) phthalate (DEHP): their interactive potential. Masters Thesis.

Ochs, J.B. 1990. Biological interaction potential of peroxisome proliferating agents in B6C3F1 mice. Masters Thesis.

Salimi, A.J. 1989. The use of morphometric analysis to evaluate hepatic peroxisome proliferation in rainbow trout (Salmo gairdneri). Masters Thesis.

Yang, J.-H. 1989. Evaluation of epigenetic carcinogens in rainbow trout by assessing peroxisome proliferation potential. Ph.D. Thesis.